ENZYMATIC PROCESSES FOR THE PRODUCTION OF 4-SUBSTITUTED 3-HYDROXYBUTYRIC ACID DERIVATIVES AND VICINAL CYANO, HYDROXY SUBSTITUTED CARBOXYLIC ACID ESTERS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation in part of U.S.S.N. 10/639,159, filed August 11, 2003, which claims the benefit under 35 U.S.C. § 119(e) of U.S.S.N. 60/402,436, filed August 9, 2002, both of which are incorporated herein by reference in their entireties.

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FIELD OF THE INVENTION

The present invention relates to novel enzymatic methods and compositions for preparing 4-substituted 3-hydroxybutyric acid derivatives and vicinal cyano, hydroxy substituted carboxylic acid esters.

BACKGROUND

4-substituted 3-hydroxybutyric acid derivatives and vicinal cyano, hydroxy substituted carboxylic acid esters are commercially important intermediates in the synthesis of pharmaceuticals. Nonracemic chiral 4-substituted 3-hydroxybutyric acid esters may be utilized in the synthesis of HMG-CoA reductase inhibitors, such as atorvastatin, fluvastatin, rosuvastatin, and itavastatin. For example, an ester of (R)-4-cyano-3-hydroxybutyric acid and an ester of (3R,5R)-6-cyano-3,5-dihydroxyhexanoic acid are key intermediates for the production of the cholesterol lowering agent atorvastatin. Methods have been described for producing certain 4-substituted 3-hydroxybutyric acid esters. Isbell, et al., Carbohydrate Res., 72:301 (1979), report a method for synthesizing an (R)-4-cyano-3-hydroxybutyric acid

ester by reacting the monohydrate calcium salt of threonine with hydrogen bromide to produce a dibromo derivative of threonine, which is then converted to a vicinal bromohydrin. The hydroxyl group of the bromohydrin is protected prior to reaction with sodium cyanide. Id.

Acta Chem. Scand., B37, 341 (1983) reports a method for producing a 4-cyano-3-hydroxybutyrate from a 4-bromo-3-hydroxybutyrate that requires protecting the hydroxy group with a protecting group prior to reaction with sodium cyanide. Recent routes to synthesize 4-cyano-3-hydroxybutyrate esters involve the uncatalyzed chemical reaction of a 4-bromo- or 4-chloro- 3-hydroxybutyrate ester, without protection of the hydroxyl group, with a cyanide salt. By-products, however, are formed under the basic conditions created by the basic cyanide anion, which are particularly problematic to remove from the product. 4-Cyano-3-hydroxybutyrate esters are high boiling liquids and vacuum fractional distillation is required to separate the 4-cyano-3-hydroxybutyrate ester from these by-products. The distillation conditions are prone to generate additional by-products and the distillation is troublesome to operate successfully.

The use of a 4-chloro-3-hydroxybutyric acid ester as a starting material in the synthesis of a 4-cyano-3-hydroxybutyric acid ester is more economically attractive than the use of a 4-bromo-3-hydroxybutyric acid ester, but requires more forcing conditions in its reaction with cyanide salts due to the lower reactivity of the chloro substituent compared to the bromo substituent. While the cyanation of 4-chloro-3-hydroxybutyrate esters proceeds with alkali cyanide and high temperature, these forcing conditions lead to substantial by-product formation, requiring extensive isolation and purification procedures that result in additional yield loss. U.S. Pat. No. 5,908,953 discloses that, besides unreacted starting material, crude lower alkyl esters of (R)-4-cyano-3-hydroxybutyric acid may contain hydroxyacrylate, cyanoacrylate, 3-cyanobutyrolactone, 3-hydroxybutyrolactone, γ-crotonolactone, 3-cyano-4-hydroxybutyrate lower alkyl ester, 3,4-dicyanobutyrate lower alkyl ester and high-boiling uncharacterized compounds. U.S. Pat. No. 5,908,953 further describes a purification method for lower alkyl esters of (R)-4-cyano-3-hydroxybutyric acid that involves distillation of a crude mixture in the presence of a solvent that has a boiling point of 50°C to 160°C at 10 Torr. Using such distillation methods, the decomposition of unreacted starting material is said to be minimized, which otherwise can result in a dramatic overall loss in (R)-4-cyano-3-hydroxybutyric acid lower alkyl ester production. U.S. Pat. No.

6,140,527 describes an alternative approach for treating crude lower alkyl esters of (R)-4-cyano-3-hydroxybutyric acid that involves removal of the dehydrated by-products, such as 4-hydroxycrotonic acid esters, by chemical reaction, which renders these components water soluble and extractable. Thus, although these methods utilize a readily available starting material, significant yield loss and product purification requirements make them commercially undesirable. Accordingly, more efficient methods for producing nonracemic chiral 4-substituted 3-hydroxybutyric acid esters under milder conditions would be highly desirable.

Halohydrin dehalogenases, also referred to as haloalcohol dehalogenases or halohydrin hydrogen-halide lyases, catalyze the elimination of hydrogen halide, as proton and halide ion, from vicinal halohydrins to produce the corresponding epoxide. These enzymes also catalyze the reverse reaction. Nagasawa et al., Appl. Microbiol. Biotechnol. vol. 36 (1992) pp. 478-482, disclose activity of a certain halohydrin hydrogen-halide lyase on 4-chloro-3-hydroxybutyronitrile among other vicinal halohydrins. Nakamura et al., Biochem. Biophys. Research Comm. vol. 180 (1991) pp. 124-130 and Tetrahedron vol. 50 (1994) pp 11821-11826, disclose activity of a halohydrin hydrogen-halide lyase to catalyze the reaction of certain epoxides with cyanide to form the corresponding beta-hydroxynitriles. In these references and U.S. Patent 5,210,031, Nakamura et al. disclose a reaction of epihalohydrin with alkali cyanide in the presence of a certain halohydrin hydrogen-halide lyase to produce the corresponding 4-halo-3-hydroxy-butyronitrile. In U.S. Patent No. 5,166,061, Nakamura et al. disclose a reaction of a 1,3-dihalo-2-propanol with alkali cyanide in the presence of certain dehalogenating enzymes to produce the corresponding 4-halo-3-hydroxybutyronitrile. In Tetrahedron vol. 50 (1994) pp 11821-11826, Nakamura et al. disclose the reaction of 1,3-dichloro-2-propanol with cyanide using a purified halohydrin hydrogen-halide lyase to produce 4-chloro-3-hydroxybutyronitrile.

Lutje-Spelberg et al., Org. Lett., vol. 2 (2001) pp 41-43, discloses activity of a halohydrin dehalogenase to catalyze the reaction of certain styrene oxides with azide to form the corresponding 1-phenyl-2-azido-ethanol.

SUMMARY OF THE INVENTION

In one aspect, the present invention is directed to a method for producing a 4-cyano-3-hydroxybutyric acid ester or amide from a 4-halo-3-hydroxybutyric acid ester or amide, the method comprising:

(a) providing a 4-halo-3-hydroxybutyric acid ester or amide,

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the 4-halo-3-hydroxybutyric acid ester or amide with a halohydrin dehalogenase and cyanide under conditions sufficient to form a reaction mixture for converting the 4-halo-3-hydroxybutyric acid ester or amide to a 4-cyano-3-hydroxybutyric acid ester or amide.

In a further aspect of the present invention, the 4-halo-3-hydroxybutyric acid ester or amide in step (a) is provided by a method comprising:

providing a 4-halo-3-ketobutyric acid ester or amide,

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

contacting the 4-halo-3-ketobutyric acid ester or amide with a ketoreductase, a cofactor, and a cofactor regeneration system under conditions sufficient to form a reaction mixture for converting the 4-halo-3-ketobutyric acid ester or amide to the 4-halo-3-hydroxybutyric acid ester or amide.

In another aspect, the present invention is directed to a method for producing a 4-cyano-3-hydroxybutyric acid ester from a 4-halo-3-ketobutyric acid ester, the method comprising:

(a) providing a 4-halo-3-ketobutyric acid ester,

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the 4-halo-3-ketobutyric acid ester with a ketoreductase, a cofactor, a cofactor regeneration system, cyanide, and a halohydrin dehalogenase to form a reaction mixture for converting the 4-halo-3-ketobutyric acid ester to a 4-cyano-3-hydroxybutyric acid ester.

In another embodiment, the present invention is directed to a method for producing a 4-nucleophile substituted-3-hydroxybutyric acid ester or amide from a 4-halo-3-hydroxybutyric acid ester or amide, the method comprising:

(a) providing a 4-halo-3-hydroxybutyric acid ester or amide,

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the 4-halo-3-hydroxybutyric acid ester or amide with a halohydrin dehalogenase and a nucleophile under conditions suitable to form a reaction mixture for converting the 4-halo-3-hydroxybutyric acid ester or amide to a 4-nucleophile substituted-3-hydroxybutyric acid or amide.

In a further embodiment, the present invention is directed to a method for producing a 4-nucleophile substituted-3-hydroxybutyric acid esters or amide, the method comprising:

(a) providing a 4-halo-3-ketobutyric acid ester or amide

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the 4-halo-3-ketobutyric acid ester or amide with a ketoreductase, a cofactor, a cofactor regeneration system, a nucleophile, and a halohydrin dehalogenase to form a reaction mixture for converting the 4-halo-3-ketobutyric acid ester or amide to a 4-nucleophile substituted-3-hydroxybutyric acid ester or amide.

In another aspect, the present invention is directed to a composition comprising:

(a) a halohydrin dehalogenase;

(b) a nucleophile; and

(c) a 4-halo-3-hydroxybutyric acid ester or amide.

The present invention further provides compositions that are useful in the production of vicinal cyano, hydroxy substituted carboxylic acid esters , as well as additional related methods for converting vicinal halo, hydroxy substituted carboxylic acid esters to vicinal cyano, hydroxy substituted carboxylic acid esters.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the amounts of ethyl 4-chloro-3-hydroxybutyrate (chlorohydrin) and ethyl 4-cyano-3-hydroxybutyrate (cyanohydrin) analyzed in test reactions of ethyl 4-chloro-

3-hydroxybutyrate with cyanide in aqueous solutions at various pHs in the presence or absence of a halohydrin dehalogenase (HHDH), as described in Example 21.

Figure 2 depicts a 3944 bp expression vector (pCK110700) of the present invention comprising a p15A origin of replication (p15 ori), a lacI repressor, a T5 promoter, a T7 ribosomal binding site (T7g10), and a chloramphenicol resistance gene (camR).

Figure 3 depicts a 4036 bp expression vector (pCK110900) of the present invention comprising a p15A origin of replication (p15 ori), a lacI repressor, a CAP binding site, a lac promoter (lac), a T7 ribosomal binding site (T7g10 RBS), and a chloramphenicol resistance gene (camR).

Figure 4 depicts the percent conversion vs. time for the reactions of ethyl (S)-4-chloro-3-hydroxybutyrate with aqueous hydrocyanic acid in the presence of various halohydrin dehalogenase enzymes that are described in Examples 25 through 29.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides enzymatic methods and compositions for producing various 4-substituted 3-hydroxybutyric acid esters and amides from corresponding 4-halo-3-hydroxybutyric acid ester and amide substrates. The present invention also provides methods and compositions for producing vicinal cyano, hydroxy substituted carboxylic acid esters from vicinal halo, hydroxy substituted carboxylic acids.

I. HALOHYDRIN DEHALOGENASE-CATALYZED CONVERSION OF 4-HALO-3-HYDROXYBUTYRIC ACID DERIVATIVES

The present invention provides a method for producing a 4-nucleophile substituted-3-hydroxybutyric acid ester or amide from a 4-halo-3-hydroxybutyric acid ester or amide, the method comprising:

(a) providing a 4-halo-3-hydroxybutyric acid ester or amide,

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the 4-halo-3-hydroxybutyric acid ester or amide with a halohydrin dehalogenase and a nucleophile under conditions suitable to form a reaction mixture for converting the 4-halo-3-hydroxybutyric acid ester or amide to a 4-nucleophile substituted-3-hydroxybutyric acid ester or amide. Significantly, the invention method provides a process

for the manufacture of 4-substituted 3-hydroxybutyric acid esters and amides in which by-product formation is minimized.

Nucleophiles suitable for use in the practice of the present invention are those that are capable of displacing the halo substituent of the 4-halo-3-hydroxybutyric acid ester or amide substrate. Typical nucleophiles utilized in the present invention are anionic nucleophiles. Exemplary nucleophiles include cyanide (CN^-), azide (N_3^-), and nitrite (ONO^-).

In a specific embodiment, the present invention provides a method for producing 4-cyano-3-hydroxybutyric acid esters or amides from 4-halo-3-hydroxybutyric acid esters or amides via a halohydrin dehalogenase-catalyzed reaction, the method comprising:

(a) providing a 4-halo-3-hydroxybutyric acid ester or amide;

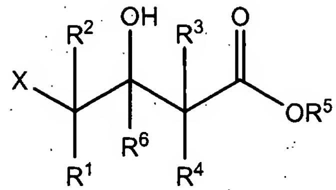
wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the 4-halo-3-hydroxybutyric acid ester or amide with a halohydrin dehalogenase and cyanide under conditions suitable to form a reaction mixture for converting the 4-halo-3-hydroxybutyric acid ester or amide to a 4-cyano-3-hydroxybutyric acid ester or amide.

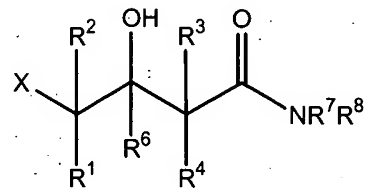
As used herein, the term "cyanide" refers to cyanide anion (CN^-), hydrocyanic acid (HCN), and mixtures thereof. Cyanide may be provided in the form of a cyanide salt, typically an alkali salt (for example, NaCN, KCN, and the like), in the form of hydrocyanic acid (gaseous or in solution), or mixtures thereof.

4-halo-3-hydroxybutyric acid esters and amides employed in the practice of the present invention may be prepared according to the methods described herein, or alternatively, using methods that are well known to those having ordinary skill in the art. Such methods are described, for example, in U.S. Patent No. 5,891,685; Hallinan, et al., Biocatalysis and Biotransformation, 12:179-191 (1995); Russ. Chem. Rev., 41:740 (1972); Kataoka, et al., Appl. Microbiol. Biotechnol., 48:699-703 (1997); and U.S. Patent No. 5,430,171.

Suitable 4-halo-3-hydroxybutyric acid ester and amide substrates employed in the practice of the present invention include those having the structure IA and 1B, respectively:



IA



IB

wherein:

X is a halogen selected from the group consisting of chlorine, bromine, and iodine;

R^1, R^2, R^3, R^4, and R^6 are each independently selected from the group consisting of hydrogen, fluorine, an optionally substituted lower alkyl, an optionally substituted cycloalkyl, an optionally substituted lower alkenyl, an optionally substituted aryl, an optionally substituted arylalkyl, amino, an optionally substituted lower alkylamino, an optionally substituted cycloalkylamino, an optionally substituted lower alkoxy, an optionally substituted cycloalkoxy, an optionally substituted aryloxy, and an optionally substituted arylalkoxy; and

R^5 is selected from the group consisting of an optionally substituted lower alkyl, an optionally substituted cycloalkyl, an optionally substituted aryl, and an optionally substituted arylalkyl; and

R^7 and R^8 are each independently selected from the group consisting of hydrogen, an optionally substituted lower alkyl, an optionally substituted cycloalkyl, an optionally substituted aryl, and an optionally substituted arylalkyl.

"Optionally substituted" refers herein to the replacement of hydrogen with a monovalent radical. Suitable substitution groups include, for example, hydroxyl, alkyl, a lower alkyl, an alkoxy, a lower alkoxy, an alkenyl, a lower alkenyl, nitro, amino, cyano, halogen (i.e., halo), thio, and the like. Other suitable substitution groups include carboxy (i.e. a carboxylate or carboxylic acid group), carboalkoxy (i.e. an ester group), carbamide (i.e.an amide group), and acyl (i.e. forming a ketone),

The term "lower alkyl" is used herein to refer to branched or straight chain alkyl groups having from one to about six carbon atoms that are unsubstituted or substituted, e.g., with one or more halo, hydroxyl or other groups, including, e.g., methyl, ethyl, propyl, isopropyl, *n*-butyl, *i*-butyl, *t*-butyl, trifluoromethyl, and the like. The term "cycloalkyl" refers to carbocylic alkyl moieties having from 3 to about 6 carbon atoms, as well as heterocyclic alkyl moieties having from 3 to about 6 atoms, where at least one ring atom is a heteroatom,

and the other atoms are carbon atoms. "Heteroatom" refers herein to oxygen, nitrogen, or sulfur.

The term "lower alkenyl" is used herein to refer to a branched or straight chain group having one or more double bonds and from 2 to about 6 carbon atoms. Lower alkenyl groups employed in the practice of the present invention may be optionally substituted with the groups described herein, including, for example, halo, hydroxyl, lower alkyl, and the like.

As used herein, the term "lower alkoxy" refers to –OR where R is a lower alkyl or a lower alkenyl. Suitable lower alkoxy groups employed in the practice of the present invention include methoxy, ethoxy, *t*-butoxy, trifluoromethoxy, and the like. The term "aryloxy" refers herein to RO-, where R is an aryl. As used herein, the term "aryl" refers to monocyclic and polycyclic aromatic groups having from 3 to about 14 backbone carbon or heteroatoms, and includes both carbocyclic aryl groups and heterocyclic aryl groups. Carbocyclic aryl groups are aryl groups in which all ring atoms in the aromatic ring are carbon. Heterocyclic aryl groups are aryl groups that have from 1 to about 4 heteroatoms as ring atoms in an aromatic ring with the remainder of the ring atoms being carbon atoms. Exemplary aryl groups employed as substituents in the present invention include, for example, phenyl, pyridyl, pyrimidinyl, naphthyl, and the like.

The term "arylalkyl" refers herein to an alkyl group substituted with an aryl group. Exemplary arylalkyl groups include benzyl, picolyl, and the like. Substituted arylalkyl groups may be substituted in either or both aryl and alkyl portions of the arylalkyl group. As used herein, the term "arylalkoxy" refers to RO- where R is an arylalkyl.

The term "cycloalkoxy" refers herein to RO-, where R is an optionally substituted C_3-C_8 cycloalkyl. The term "amino" is used herein to refer to the group $-NH_2$. The term "lower alkylamino" refers herein to the group –NRR' where R is hydrogen or a lower alkyl, and R' is a lower alkyl. The term "cycloalkylamino" refers herein to the group –NR where R is an optionally substituted divalent aliphatic radical having from 3 to about 8 carbon atoms, so that N and R form a cyclic structure, for example, pyrollidino, piperidino, and the like.

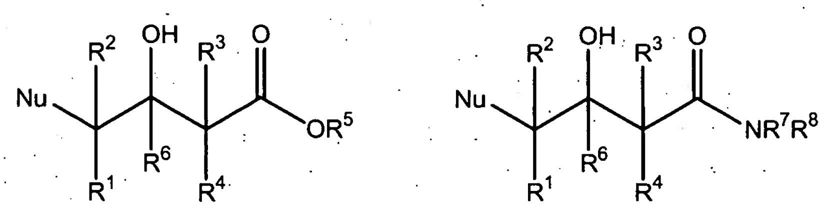
Specific 4-halo-3-hydroxybutyric acid esters of compound IA that may be employed in the practice of the present invention include ethyl 4-chloro-3-hydroxybutyric acid ester (i.e., where X is chlorine, R^1, R^2, R^3, R^4, and R^6 are hydrogen, and R^5 is ethyl), methyl 4-chloro-3-hydroxybutyric acid ester (i.e., where X is chlorine, R^1, R^2, R^3. R^4 and R^6 are hydrogen and R^5 is methyl), ethyl 4-bromo-3-hydroxybutyric acid ester (i.e., where X is

bromine, R^1, R^2, R^3, R^4, and R^6 are hydrogen, and R^5 is ethyl), methyl 4-bromo-3-hydroxybutyric acid ester (i.e., where X is bromine, R^1, R^2, R^3, R^4, and R^6 are hydrogen, and R^5 is methyl), t-butyl-4-chloro-3-hydroxybutyric acid ester (i.e., where X is chlorine, R^1, R^2, R^3, R^4, and R^6 are hydrogen, and R^5 is t-butyl), t-butyl-4-bromo-3-hydroxybutyric acid ester (i.e., where X is bromine, R^1, R^2, R^3, R^4, and R^6 are hydrogen, and R^5 is t-butyl), and t-butyl-4-iodo-3-hydroxybutyric acid ester (i.e., where X is iodine, R^1, R^2, R^3, R^4, and R^6 are hydrogen, and R^5 is t-butyl). In certain embodiments, at least one of R^1, R^2, R^3, R^4, and R^6 is a lower alkyl, such as, for example, methyl, ethyl, or propyl.

Suitable 4-halo-3-hydroxybutyric acid amides of compound IB that may be employed in the practice of the present invention include 4-chloro-3-hydroxybutyric amide (i.e., where X is chlorine, R^1, R^2, R^3, R^4, R^6, R^7, and R^8 are hydrogen), 4-bromo-3-hydroxybutyric amide (i.e., where X is bromine, and R^1, R^2, R^3, R^4, R^6, R^7, and $R^8$are hydrogen), and 4-iodo-3-hydroxybutyric amide (i.e., where X is iodine, R^1, R^2, R^3, R^4, R^6, R^7, and R^8 are hydrogen, In certain embodiments, at least one of R^1, R^2, R^3, R^4, and R^6 is a lower alkyl, such as, for example, methyl, ethyl, or propyl.

The 4-halo substituent of the 4-halo-3-hydroxybutyric acid ester and amide substrates is preferably selected from chlorine and bromine. Particularly preferred are 4-chloro-3-hydroxybutyric acid ester and amide substrates.

4-substituted-3-hydroxybutyric acid esters and amides produced by the methods of the present invention include those having the structure IIA and IIB, respectively:



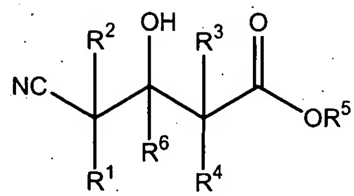
IIA IIB

where:

R^1, R^2, R^3, R^4, R^5, R^6, R^7, and R^8 are as defined for structures IA and IB; and

Nu is selected from the group consisting of –CN, $–N_3$, and –ONO.

When 4-halo-3-hydroxybutyric acid ester substrates having the structure of compound IA are reacted with cyanide and halohydrin dehalogenase, 4-cyano-3-hydroxybutyric acid ester products are generated that have the structure of compound III:



III

where R^1, R^2, R^3, R^4, R^5, and R^6 are as defined for structure IA .

Halohydrin dehalogenases are employed in the practice of the present invention to catalyze the conversion of a 4-halo-3-hydroxybutyric acid ester or amide to the corresponding 4-nucleophile substituted-3-hydroxybutyric acid ester or amide in the presence of a nucleophile. The terms "halohydrin dehalogenase" and "HHDH" are used interchangeably herein to refer to an enzyme that, in the process of the present invention, catalyzes the conversion of a vicinal halo, hydroxy substituted carboxylic ester or amide to a vicinal cyano, hydroxyl substituted carboxylic ester or amide, such as, for example, 4-halo-3-hydroxybutyric acid ester and/or amide to a 4-nucleophile substituted-3-hydroxybutyric acid ester and/or amide, respectively, in the presence of a nucleophile such as cyanide. Suitable halohydrin dehalogenases include naturally occurring (wild type) halohydrin dehalogenases, as well as non-naturally occurring halohydrin dehalogenases generated by human manipulation. Exemplary naturally occurring and non-naturally occurring halohydrin dehalogenases and halohydrin dehalogenase-encoding polynucleotides include those described herein.

Naturally occurring halohydrin dehalogenase encoding genes have been identified in *Agrobacterium radiobacter* AD1 (*hheC*), *Agrobacterium tumefaciens (halB)*, *Corynebacterium sp.* (*hheA* encoding Ia and *hhB* encoding Ib), *Arthrobacter sp.* ($hheA_{AD2}$), and *Mycobacterium* sp. GP1 ($hheB_{GP1}$). See van Hylckama Vlieg, J.E.T., L. Tang, J.H. Lutje Spelberg, T. Smilda, G.J. Poelarends, T. Bosma, A.E.J. van Merode, M.W. Fraaije & Dick B. Janssen, "Halohydrin Dehalogenases are structurally and mechanistically related to short-chain dehydrogenases/reductases (2001) Journal of Bacteriology, 183:5058-5066 (provides the amino acid sequences for these halohydrin dehalogenases in an alignment).

These naturally occurring halohydrin dehalogenases have been characterized to some extent. HHDH from *Agrobacterium radiobacter* AD1 is a homotetramer of 28 kD subunits.

Corynebacterium sp. N-1074 produces two HHDH enzymes, one of which is composed of 28 kD subunits (Ia), while the other is composed of related subunits of 35 and/or 32 kD (Kb). HHDH from some sources is easily inactivated under oxidizing conditions in a process that leads to dissociation of the subunits, has a broad pH optimum from pH 8 to 9 and an optimal temperature of 50°C (Tang, Enz. Microbiol. Technol. (2002) 30:251-258; Swanson, Curr. Opinion Biotechnol. (1999) 10:365-369). The optimal pH for HHDH catalyzed epoxide formation is 8.0 to 9.0 and the optimal temperature ranges from 45 to 55°C (Van Hylckama Vlieg, et al., J. Bacteriol. (2001) 183:5058-5066; Nakamura, et al., Appl. Environ. Microbiol. (1994) 60:1297-1301; Nagasawa, et al., Appl. Microbiol. Biotechnol. (1992) 36:478-482). The optimal pH for the reverse reaction, ring opening by chloride has been reported for the two *Cornebacterium* sp. N-1074 enzymes and is 7.4 (Ia) or 5 (Ib). Polynucleotides encoding the halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 are provided herein as SEQ ID NOS: 13, 15, and 17. The polynucleotides corresponding to SEQ ID NOS: 13, 15, and 17 are variants that encode the same amino acid sequence (the translated sequences are provided as SEQ ID NOS: 14, 16, and 18).

Non-naturally occurring halohydrin dehalogenases can be generated using known methods, including, for example, mutagenesis, directed evolution, and the like. Several illustrative methods are described hereinbelow. The enzymes can be readily screened for activity using the method described in Example 4. Such screening methods may also be readily applied to identifying other naturally occurring halohydrin dehalogenases. Suitable non-naturally occurring halohydrin dehalogenases include those corresponding to SEQ ID NOS: 24 (HHDH B-03), 26 (HHDH C-04), 28 (HHDH E-01), 30 (S01056858), 32 (HHDH 2G5), 34 (HHDH Mz1.1A5), 36 (HHDH cys1.10), 38 (HHDH cys2.12), 74 (HHDH B-12), 76 (HHDH Mz1/4H6), 78 (HHDH F-04), 80 (HHDH A-08), 82 (HHDH G9), 84 (HHDH F9), 86 (HHDH H10), 88 (HHDH A1), 90 (HHDH A-03), 92 (HHDH E-03), 94 (HHDH S00827801), 96 (HHDH S00890554), 98 (HHDH S00994580), 100 (HHDH S01018044), 102 (HHDH S01035939), 104 (HHDH S01009684), 106 (HHDH S00817219), 108 (HHDH S00708827), 110 (HHDH S00772501), 112 (HHDH S01035968), and 114 (HHDH S01040430). Exemplary polynucleotide sequences that encode these halohydrin dehalogenases include those corresponding to SEQ ID NOS: 23, 25, 27, 29, 31, 33, 35, 37, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, and 113, respectively. Additional non-naturally occurring halohydrin dehalogenases that are suitable

for use in the practice of the present invention are provided in the patent application entitled, "Improved Halohydrin Dehalogenases and Related Polynucleotides," corresponding to Attorney Docket No. 0353.110US, filed on August 11, 2003, and assigned U.S. application serial number 60/494,382, and in the patent application entitled, "Improved Halohydrin Dehalogenases and Related Polynucleotides," corresponding to Attorney Docket No. 0353.210US, filed on February 18, 2004, and assigned U.S. application serial number ____________________, both of which are incorporated herein by reference in their entireties.

Halohydrin dehalogenases that are suitable for use in the practice of the present invention, whether naturally occurring or non-naturally occurring, can be readily identified by those having ordinary skill in the art using the method described in Example 4 and the vicinal halo, hydroxyl substituted substrate and nucleophile of interest.. Halohydrin dehalogenases employed in the practice of the present invention typically exhibit an activity of at least about 1 μmol/min/mg in the assay described in Example 4. Halohydrin dehalogenases employed in the practice of the present invention may exhibit an activity of at least about 10 μmol/min/mg, and sometimes at least about 10^2 μmol/min/mg, and up to about 10^3 μmol/min/mg or higher, in the assay described in Example 4.

Halohydrin dehalogenase may be provided to the reaction mixture in the form of purified enzyme, cell extract, cell lysate, or whole cells transformed with gene(s) encoding halohydrin dehalogenase(s). Whole cells transformed with halohydrin dehalogenase encoding genes and/or cell extracts and/or cell lysates thereof may be employed in a variety of different forms, including solid (e.g., lyophilized, spray dried, and the like) or semi-solid (e.g., a crude paste). The cell extracts or cell lysates may be partially purified by precipitation (ammonium sulfate, polyethyleneimine, heat treatment or the like), followed by a desalting procedure prior to lyophilization (e.g., ultrafiltration, dialysis, and the like). Any of the cell preparations may be stabilized by crosslinking using known crosslinking agents, such as, for example, glutaraldehyde or immobilization to a solid phase (e.g., Eupergit C, and the like).

The solid reactants (e.g., enzyme, salts, etc.) may be provided in a variety of different forms, including powder (e.g., lyophilized, spray dried, and the like), solution, emulsion, suspension, and the like. The reactants can be readily lyophilized or spray dried using methods and equipment that are known to those having ordinary skill in the art. For example,

the protein solution can be frozen at -80°C in small aliquots, then added to a prechilled lyophilization chamber, followed by the application of a vacuum. After the removal of water from the samples, the temperature is typically raised to 4°C for two hours before release of the vacuum and retrieval of the lyophilized samples.

In carrying out the conversion of 4-halo-3-hydroxybutyric acid ester or amide substrate to the corresponding 4-nucleophile substituted-3-hydroxybutyric ester or amide product, the substrate is typically contacted with the halohydrin dehalogenase and nucleophile in a solvent. Suitable solvents for carrying out the conversion of 4-halo-3-hydroxybutyric acid ester or amide to 4-nucleophile substituted-3-hydroxybutyric acid ester or amide include water, organic solvents (e.g. ethyl acetate, butyl acetate, 1-octanol, heptane, octane, methyl t-butyl ether (MTBE), toluene, and the like), ionic liquids (e.g., 1-ethyl 4-methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium hexafluorophosphate, and the like), and co-solvent systems, including aqueous co-solvent systems, and the like. Preferred solvents are aqueous solvents, including water and aqueous co-solvent systems.

Exemplary aqueous co-solvent systems have water and one or more organic solvent. In general, an organic solvent component of an aqueous co-solvent system is selected such that it does not completely inactivate the enzyme catalysts employed in the invention method. Appropriate co-solvent systems can be readily identified by measuring enzyme activity with the substrate of interest in the candidate solvent system, utilizing the enzyme assay described in Example 4.

The organic solvent component of an aqueous co-solvent system may be miscible with the aqueous component, providing a single liquid phase, or may be partly miscible or immiscible with the aqueous component, providing two liquid phases. Typically, when an aqueous co-solvent system is employed, it is selected to be biphasic, with water dispersed in an organic solvent, or vice-versa. Generally, when an aqueous co-solvent system is utilized, it is desirable to select an organic solvent that can be readily separated from the aqueous phase. In general, the ratio of water to organic solvent in the co-solvent system is typically in the range of from about 90:10 to about 10:90 (v/v) organic solvent to water, and between 80:20 and 20:80 (v/v) organic solvent to water. The co-solvent system may be pre-formed prior to addition to the reaction mixture, or it may be formed *in situ* in the reaction vessel.

The aqueous solvent (water or aqueous co-solvent system) may be pH-buffered or unbuffered. The conversion of the 4-halo-3-hydroxybutyric acid ester or amide to the 4-nucleophile substituted-3-hydroxybutyric acid ester or amide may be carried out at a pH of about 5 or above. Generally, the conversion is carried out at a pH of about 10 or below, usually in the range of from about 5 to about 10.. Typically, the conversion is carried out at a pH of about 9 or below, usually in the range of from about 5 to about 9. Preferably, the conversion is carried out at a pH of about 8 or below, usually in the range of from about 5 to about 8, and more preferably in the range of from about 6 to about 8. This conversion may also be carried out at a pH of about 7.8 or below, or 7.5 or below. Alternatively, the conversion may be carried out a neutral pH, i.e., about 7.

During the course of conversion, the pH of the reaction mixture may change. The pH of the reaction mixture may be maintained at a desired pH or within a desired pH range by the addition of an acid or a base during the course of conversion. Alternatively, the pH change may be controlled by using an aqueous solvent that comprises a buffer. Suitable buffers to maintain desired pH ranges are known in the art and include, for example, phosphate buffer, triethanolamine buffer, and the like. Combinations of buffering and acid or base addition may also be used.

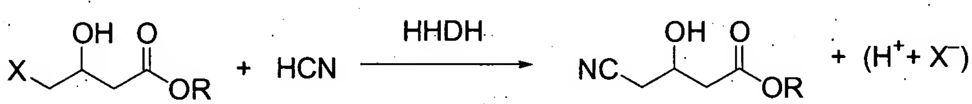
As described above, when conversion to 4-cyano-3-hydroxybutyric acid derivative is desired, the cyanide may be provided in the form of a cyanide salt, typically an alkali salt (for example, NaCN, KCN, and the like), in the form of hydrocyanic acid (gaseous or in solution), or mixtures thereof. Hydrocyanic acid is a weak acid. In aqueous solutions within several pH units of its pKa (pKa = 9.1 in water) cyanide is present as both CN^- and HCN in equilibrium concentrations. At pH values below about 9, cyanide is predominantly present as HCN.

When the cyanide is provided by a cyanide salt, the reaction mixture is typically buffered or acidified or both to provide the desired pH. Suitable acids for acidification of basic cyanide salts solutions include organic acids, for example carboxylic acids, sulfonic acids, phosphonic acids, and the like, mineral acids, for example hydrohalic acids (such as hydrochloric acid), sulfuric acid, phosphoric acid, and the like, acidic salts, for example dihydrogenphosphate salts (e.g. KH_2PO_4), bisulfate salts (e.g. $NaHSO_4$) and the like, as well as hydrocyanic acid. The acids or acid salts used to acidify the cyanide salt may be selected to also provide a buffer in the resulting solution. For example, acidification with phosphoric

acid or a dihydrogenphosphate salt may be used to provide a phosphate buffered solution of HCN in the phosphate buffer range (about pH 6-8).

When the cyanide is provided by hydrocyanic acid and a higher pH than that so created is desired, the reaction mixture is typically buffered or made less acidic by adding a base to provide the desired pH. Suitable bases for neutralization of hydrocyanic acid are organic bases, for example amines, alkoxides and the like, and inorganic bases, for example, hydroxide salts (e.g. NaOH), carbonate salts (e.g. $NaHCO_3$), bicarbonate salts (e.g. K_2CO_3), basic phosphate salts (e.g. K_2HPO_4, Na_3PO_4), and the like, as well as cyanide salts.

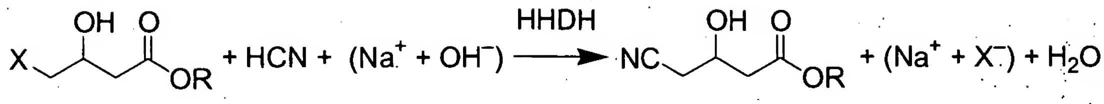
For pH values below about 9, at which cyanide is predominantly present as HCN, equation (1) describes the halohydrin dehalogenase catalyzed reaction of a 4-halo-3-hydroxybutyric acid ester with the HCN in unbuffered aqueous reaction mixtures.



(1)

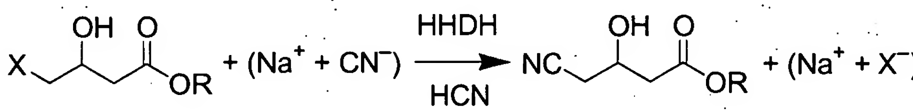
The consumption of the hydrocyanic acid, a weak acid (pKa ~9) and release of the hydrohalic acid, a strong acid (pKa <0), causes the pH of the reaction mixture to drop if the aqueous hydrohalic acid ($H^+ + X^-$) is not otherwise neutralized. The pH of the reaction mixture may be maintained at the desired level by standard buffering techniques, wherein the buffer neutralizes the hydrohalic acid up to the buffering capacity provided, or by the addition of a base concurrent with the course of the conversion. Such addition may be done manually while monitoring the reaction mixture pH or, more conveniently, by using an automatic titrator as a pH stat. A combination of partial buffering capacity and base addition can also be used for process control.

When the pH is maintained by buffering or by addition of a base over the course of the conversion, an aqueous halide salt rather than aqueous hydrohalic acid is the product of the overall process. For example, equation (2) represents the overall process when aqueous sodium hydroxide ($Na^+ + OH^-$) is added over the course of the reaction to maintain an initial pH below about 9.



(2)

In the embodiment wherein a cyanide salt is added as the base to neutralize the hydrohalic acid as it is produced, the neutralization regenerates HCN and maintains the total cyanide concentration ($HCN + CN^-$) as well as the pH in the reaction mixture. This can be advantageous if the rate of conversion otherwise decreases as cyanide concentration decreases. For example, equation (3) represents the overall process when aqueous sodium cyanide ($Na^+ + CN^-$) is added over the course of the reaction to maintain an initial pH. While the cyanide is present predominantly as HCN in the reaction mixture, the HCN concentration is maintained while the conversion in net consumes the added basic cyanide salt.



(3)

When base addition is employed to neutralize the hydrohalic acid released during the halohydrin dehalogenase-catalyzed reaction of a 4-halo-3-hydroxybutyrate ester or amide to a 4-cyano-3-hydroxybutyric acid ester or amide, the progress of the conversion may be monitored by the amount of base added to maintain the pH. Typically bases added to unbuffered or partially buffered reaction mixtures over the course of conversion are added in aqueous solutions.

When the nucleophile is the conjugate anion of a stronger acid, having a pKa significantly below the initial pH of the reaction solution, the nucleophile is present predominantly in its anionic form so that, unlike with HCN, a proton is not released on its reaction. Accordingly, the reaction mixture pH in reactions of such nucleophiles may be maintained without stoichiometric buffering or base addition. For example, the conjugate acid of azide, hydrazoic acid has a pKa of 4.7 and the conjugate acid of nitrite, nitrous acid, has a pKa of 3.3. Accordingly, at neutral pH, these nucleophiles are present predominantly in their anionic form, N_3^- and ONO^-, respectively. That is, the neutral reaction mixture comprises aqueous azide and nitrite salt, respectively. Their reaction in such mixtures releases halide anion to form aqueous halide salt, not aqueous hydrohalic acid.

Those having ordinary skill in the art can readily determine the quantities of HHDH, 4-halo-3-hydroxybutyric acid ester or amide substrate and nucleophile to use based on, for example, the activity of HHDH as determined by the method in Example 4, the quantity of product desired, and the like. To illustrate, the amount of 4-halo-3-hydroxybutyric acid ester or amide can be in the range of from about 10 to about 500 g/L using about 10 mg to about 30 g of halohydrin dehalogenase. The stoichiometric amount of nucleophile can be readily determined. Further illustrative examples are provided herein.

Suitable conditions for carrying out the HHDH-catalyzed conversion of the present invention include a wide variety of conditions which can be readily optimized by routine experimentation that includes contacting the HHDH, 4-halo-3-hydroxybutyric acid ester or amide substrate, and nucleophile at an experimental pH and temperature and detecting product, for example, using the methods described in the Examples provided herein. The HHDH-catalyzed conversion of 4-halo-3-hydroxybutyric acid ester or amide to 4-nucleophile substituted-3-hydroxybutyric acid ester or amide is typically carried out at a temperature in the range of from about 15°C to about 75°C. More typically, the reaction is carried out at a temperature in the range of from about 20°C to about 55°C, and typically from about 20°C to about 45°C. The reaction may also be carried out under ambient conditions.

The HHDH-catalyzed conversion of 4-halo-3-hydroxybutyric acid ester or amide to 4-nucleophile substituted-3-hydroxybutyric acid ester or amide is generally allowed to proceed until essentially complete or near complete conversion of substrate. Conversion of substrate to product can be monitored using known methods by detecting substrate and/or product. Suitable methods include gas chromatography, HPLC, and the like. Yields of the 4-nucleophile substituted-3-hydroxybutyric acid ester or amide generated in the reaction mixture are generally greater than about 50%, may also be greater than about 60%, may also be greater than about 70%, may be also be greater than about 80%, and are often greater than about 90%.

The 4-nucleophile substituted-3-hydroxybutyric acid ester or amide may be collected from the reaction mixture and optionally purified using methods that are known to those having ordinary skill in the art, as well as those described in the Examples.

Preferred 4-halo-3-hydroxybutyric acid ester or amide substrates of the present invention are chiral, being stereogenic at the 3-position, and may be racemic or non-racemic. Certain halohydrin dehalogenase enzymes used in the process of the present invention

convert the chiral substrate to the 4-cyano-3-hydroxybutyric acid ester or amide with retention of the absolute stereochemistry at the stereogenic 3-position. Non-racemic chiral 4-halo-3-hydroxybutyric acid ester or amide substrates may be converted to substantially equally non-racemic 4-cyano-3-hydroxybutyric acid ester or amide products with little or no loss in stereopurity. The Examples show embodiments of the invention providing high retention of enantiopurity. (Due to conventions for designating stereochemistry, the enantiomer of ethyl 4-chloro-3-hydroxybutyrate designated as (S) and the enantiomer ethyl 4-cyano-3-hydroxybutyrate designated as (R) have the identical stereoconfiguration at the 3-position.)

In other embodiments of the present invention, certain halohydrin dehalogenase enzymes may be stereospecific for one stereoisomer of the chiral 4-halo-3-hydroxybutyric acid ester or amide substrate. The process of the present invention using such stereospecific enzymes may be used to react one stereoisomer of a stereoisomeric mixture of a 4-halo-3-hydroxybutyric acid ester or amide, for example a racemic mixture, while leaving the other stereoisomer substantially unreacted, thereby providing a kinetic resolution of the mixture.

A further significant characteristic of the present invention is that the purity of the 4-nucleophile substituted-3-hydroxybutyric acid ester or amide products generated is very high without the need for extensive purification procedures such as vacuum distillation. Typically, the purity of 4-nucleophile substituted-3-hydroxybutyric acid ester or amide products generated in accordance with the methods of the present invention are at least about 80%, usually at least about 90%, and typically at least about 95%. Product purity may be determined by conventional methods such as HPLC or gas chromatography.

II. KETOREDUCTASE-CATALYZED PRODUCTION OF HALOHYDRINS

The present invention further provides an enzymatic method for generating a 4-halo-3-hydroxybutyric acid ester or amide by:

(a) providing a 4-halo-3-ketobutyric acid ester or amide,

wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

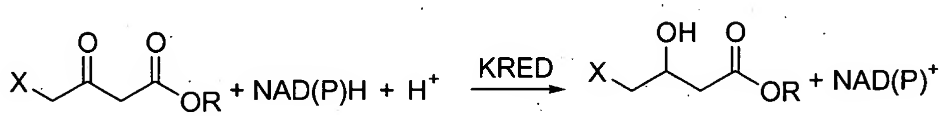
(b) contacting the 4-halo-3-ketobutyric acid ester or amide with a ketoreductase, a cofactor, and a cofactor regeneration system under conditions suitable to form a reaction

mixture for converting the 4-halo-3-ketobutyric acid ester or amide to the 4-halo-3-hydroxybutyric acid ester or amide.

The terms "ketoreductase" and "KRED" are used interchangeably herein to refer to an enzyme that, in the process of the present invention, catalyzes the reduction of a 4-halo-3-ketobutyric acid ester or amide to the corresponding 4-halo-3-hydroxybutyric acid ester or amide. Such catalytic activity may be detected in an assay such as that described in Example 4, hereinbelow.

As used herein, the term "cofactor" refers to a non-protein compound that operates in combination with an enzyme which catalyzes the reaction of interest. Suitable cofactors employed in the practice of the present invention include $NADP^+$ (nicotinamide-adenine dinucleotide phosphate), NADPH (i.e., the reduced form of nicotinamide adenine dinucleotide phosphate), NAD^+ (i.e., nicotinamide adenine dinucleotide), and NADH (i.e., the reduced form of NAD^+), and the like. The reduced form of the cofactor is regenerated by reducing the oxidized cofactor with a cofactor regeneration system.

In the present process, the ketoreductase catalyzes the reduction of the 4-halo-3-ketobutyric acid ester or amide by the reduced form of the cofactor. Equation (4) describes the ketoreductase-catalyzed reduction of a 4-halo-3-ketobutyric acid ester by NADH or NADPH, which are represented as alternatives by the designation NAD(P)H.



(4)

Ketoreductases that are suitable for carrying out the reduction of 4-halo-3-ketobutyric acid ester or amide to 4-halo-3-hydroxybutyric acid ester or amide include both naturally occurring ketoreductases, as well as non-naturally occurring ketoreductases generated by human manipulation Exemplary naturally occurring and non-naturally occurring ketoreductases and ketoreductase-encoding polynucleotides include those described herein.

Naturally occurring KRED enzymes can be found in a wide range of bacteria and yeasts. Several naturally occurring KRED gene and enzyme sequences have been reported in the literature, such as, *Candida magnoliae* (Genbank Acc. No. JC7338; GI:11360538), *Candida parapsilosis* (Genbank Ac. No. BAA24528.1; GI:2815409), *Sporobolomyces*

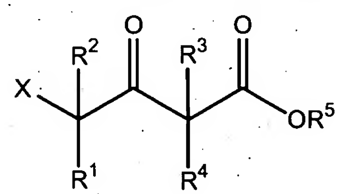
salmicolor (Genbank Acc. No. AF160799; GI 6539734). Polynucleotide sequences encoding the ketoreductase from *Candida magnoliae* are provided as SEQ ID NOS: 1 (CR2-5), 3 (CR1-2), 5 (CR1-3), and 7 (CR2-4). SEQ ID NOS: 1 (CR2-5), 5 (CR1-3), and 7 (CR2-4) are variants that encode the *C. magnoliae* protein (SEQ ID NOS: 2, 6, and 8). SEQ ID NO: 3 (CR1-2) encodes a variant that differs from the *C. magnoliae* protein by one amino acid change (SEQ ID NO: 4). Enzymatic reduction of β-keto esters has been reported for a carbonyl reductase from *Rhodococcus erythropolis* (Peters, Appl. Microbiol. Biotechnol. (1992) 38:334-340; Zelinski, J. Biotechnol. (1994) 33:283-292), an aldehyde reductase from *Sporoboromyces salmonicolor* AKU 4429 (Shimizu, Biotechnol. Lett. (1990) 12:593-596; Appl. Environ. Microbiol. (1990) 56:2374-2377). Enzymes such as those derived from *S. cerevisiae* (J. Org. Chem. (1991) 56:4778; Biosci. Biotech. Biochem. (1994) 58:2236), *Sporobolomyces salmonicolor* (Biochim. Biophys. Acta (1992) 1122:57), *Sporobolomyces sp.* (Biosci. Biotech. Biochem. (1993) 57:303; Japanese patent publication JP2566960), *Candida albicans* (Biosci. Biotech. Biochem. (1993) 57:303), *Candida macedoniensis* (Arch. Biochem. Biophys. (1992) 294-469), *Geotrichium candidum* (Enzyme Microbiol. Technol. (1992) 14:731) have been used for the reduction of ethyl 4-chloro-3-acetoacetate (ECAA). U.S. Pat. No. 6,168,935 describes the use of glycerol dehydrogenase (Tetrahedron Lett. (1988) 29:2453), alcohol dehydrogenase (ADH) from *Thermoanaerobium brockii* (JACS (1985) 107:4028), or *Sulfolobus solfataricus* (Biotechnol. Lett. (1991) 13:31) or *Pseudomonas* sp. (U.S. Pat. No. 5,385,833; J. Org. Chem. (1992) 57:1526).

Suitable non-naturally occurring ketoreductases can be readily identified by applying known methods, including mutagenesis, directed evolution, and the like, followed by screening for activity using the method described in Example 4. For example, these methods can be readily applied to naturally occurring ketoreductases, including the ones described herein. Exemplary non-naturally occurring ketoreductases are provided herein as SEQ ID NOS: 40 (KRED krh133c), 42 (KRED krh215), 44 (KRED krh267), 46 (KRED krh287), 48 (KRED krh320), 50 (KRED krh326), 52 (KRED krh408), 54 (KRED krh417), 56 (KRED krh483), 58 (KRED krh476), 60 (KRED krh495), 114 (KRED S01040430), 116 (KRED S01091361), 118 (KRED S01091625), and 120 (KRED S01094648). The polynucleotide sequences that encode them are provided herein as SEQ ID NOS: 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 113, 115, 117, and 119, respectively. Additional non-naturally occurring ketoreductases that are suitable for use in the practice of the present invention are provided in

the patent application entitled, "Improved Ketoreductase Polypeptides and Related Polynucleotides," corresponding to Attorney Docket No. 0190.110US/15077US01, filed on August 11, 2003, and assigned U.S. application serial number 60/494,195, and in the patent application entitled, "Improved Ketoreductase Polypeptides and Related Polynucleotides," corresponding to Attorney Docket No. 0190.210US/15077US02, both of which are incorporated herein by reference in their entireties.

Ketoreductases employed in the practice of the present invention typically exhibit an activity of at least about 1 μmol/min/mg in the assay described in Example 4, using the 4-halo-3-ketobutyric acid ester or amide substrate of interest. Ketoreductases employed in the practice of the present invention may exhibit an activity of at least 1 μmol/min/mg to about 10 μmol/min/mg and sometimes at least about 10^2 μmol/min/mg, up to about 10^3 μmol/min/mg or higher.

4-halo-3-ketobutyric acid esters and amides employed in the practice of the present invention can be readily purchased or synthesized using known methods. Exemplary 4-halo-3-ketobutyric acid ester substrates include those having the structure IV:



IV

where:

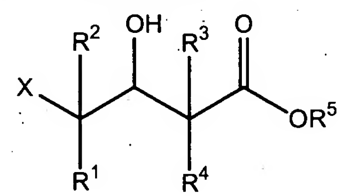
X is a halogen selected from the group consisting of chlorine, bromine, and iodine; and

R^1 , R^2, R^3, R^4, and R^5 are selected as described for structure 1A.

Specific 4-halo-3-ketobutyric acid esters that may be employed in the practice of the present invention include ethyl 4-chloro-3-ketobutyric acid ester (i.e., where X is chlorine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is ethyl), methyl 4-chloro-3-ketobutyric acid ester (i.e., where X is chlorine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is methyl), ethyl 4-bromo-3-ketobutyric acid ester (i.e., where X is bromine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is ethyl), ethyl 4-iodo-3-ketobutyric acid ester (i.e., where X is iodine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is ethyl), methyl 4-bromo-3-ketobutyric acid ester (i.e., where X is bromine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is methyl), methyl

4-iodo-3-ketobutyric acid ester (i.e., where X is iodine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is methyl), t-butyl-4-chloro-3-ketobutyric acid ester (i.e., where X is chlorine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is t-butyl), t-butyl-4-bromo-3-ketobutyric acid ester (i.e., where X is bromine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is t-butyl), and t-butyl-4-iodo-3-ketobutyric acid ester (i.e., where X is iodine, R^1, R^2, R^3, and R^4 are each hydrogen, and R^5 is t-butyl). In certain embodiments, at least one of R^1, R^2, R^3, and R^4 is a lower alkyl, such as, for example, methyl, ethyl, or propyl.

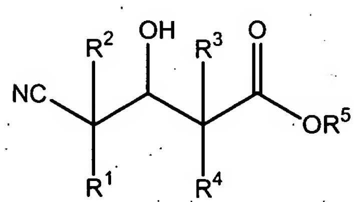
When 4-halo-3-ketobutyric acid ester substrates having the structure of compound IV are reduced during the KRED-catalyzed conversion of the present invention, 4-halo-3-hydroxybutyric acid esters are generated having the structure V:



V

where X, R^1, R^2, R^3, R^5 and R^5 are as described for structure IV.

4-halo-3-hydroxybutyric acid esters or amides produced by the ketoreductase-catalyzed reduction method of the present invention can then be readily used in the halohydrin dehalogenase-catalyzed conversions of the present invention. For example, 4-halo-3-hydroxybutyric acid esters corresponding to structure V can be used as substrate for conversion by HHDH in the presence of cyanide to generate 4-cyano-3-hydroxybutyric acid esters having the structure VI:



VI

where R^1, R^2, R^3, R^4, and R^5 are as described as for compound V.

The term "cofactor regeneration system" refers herein to a set of reactants that participate in a reaction that reduces the oxidized form of the cofactor (e.g., NADP to NADPH). Cofactors oxidized by the ketoreductase-catalyzed reduction of the 4-halo-3-ketobutyric acid ester or amide are regenerated in reduced form by the cofactor regeneration

system. Cofactor regeneration systems comprise a stoichiometric reductant that is a source of reducing hydrogen equivalents and is capable of reducing the oxidized form of the cofactor. The cofactor regeneration system may further comprise a catalyst, for example an enzyme catalyst, that catalyzes the reduction of the oxidized form of the cofactor by the reductant. Cofactor regeneration systems to regenerate NADH or NADPH from NAD or NADP, respectively, are known in the art and may be used in the present invention.

Suitable cofactor regeneration systems employed in the practice of the present invention include glucose and glucose dehydrogenase, formate and formate dehydrogenase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, isopropyl alcohol and secondary alcohol dehydrogenase, phosphite and phosphite dehydrogenase,molecular hydrogen and hydrogenase, and the like, and may be used in combination with either NADP/NADPH or NAD/NADH as the cofactor. Electrochemical regeneration using hydrogenase may also be used as a cofactor regeneration system. See, e.g., U.S. Patent Nos. 5,538,867 and 6,495,023, both of which are incorporated herein by reference. Chemical cofactor regeneration systems comprising a metal catalyst and a reducing agent (for example, molecular hydrogen or formate) are also suitable. See, e.g., PCT publication WO 2000053731, which is incorporated herein by reference.

The terms "glucose dehydrogenase" and "GDH" are used interchangeably herein to refer to an NAD or NADP-dependent enzyme that catalyzes the conversion of D-glucose and NAD or NADP to gluconic acid and NADH or NADPH, respectively. Equation (5) describes the glucose dehydrogenase-catalyzed reduction of NAD or NADP by glucose.

$$\text{glucose} + \text{NAD(P)}^+ + H_2O \xrightarrow{\text{GDH}} \text{gluconic acid} + \text{NAD(P)H} + H^+ \qquad (5)$$

Glucose dehydrogenases that are suitable for use in the practice of the present invention include both naturally occurring glucose dehydrogenases, as well as non-naturally occurring glucose dehydrogenases. Naturally occurring glucose dehydrogenase encoding genes have been reported in the literature. For example, the *Bacillus subtilis* 61297 GDH gene was expressed in *E. coli* and was reported to exhibit the same physicochemical properties as the enzyme produced in its native host (Vasantha, et al., Proc. Natl. Acad. Sci. USA (1983) 80:785). The gene sequence of the *B. subtilis* GDH gene, which corresponds to

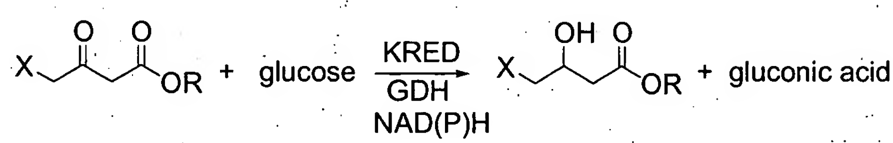
Genbank Acc. No. M12276, was reported by Lampel, et al. (J. Bacteriol. (1986) 166:238-243) and in corrected form by Yamane, et al. (Microbiology (1996) 142:3047-3056) as Genbank Acc. No. D50453. Naturally occurring GDH genes also include those that encode the GDH from *B. cereus* ATCC 14579 (Nature (2003) 423:87-91; Genbank Acc. No. AE017013) and *B. megaterium* (Eur. J. Biochem. (1988) 174:485-490, Genbank Acc. No. X12370; J. Ferment. Bioeng. (1990) 70:363-369, Genbank Acc. No. GI216270). Glucose dehydrogenases from *Bacillus sp.* are provided herein as SEQ ID NOS: 10 and 12 (encoded by polynucleotide sequences corresponding to SEQ ID NOS: 9 and 11, respectively).

Non-naturally occurring glucose dehydrogenases may be generated using known methods, such as, for example, mutagenesis, directed evolution, and the like. GDH enzymes having suitable activity, whether naturally occurring or non-naturally occurring, may be readily identified using the assay described in Example 4. Exemplary non-naturally occurring halohydrin dehalogenases are provided herein as SEQ ID NOS: 62 (GDH 2313), 64 (GDH 2331), 66 (GDH 2279), 68 (GDH 2379), 122 (GDH S01024744), 124 (GDH S01052992), and 126 (GDH S01063714). The polynucleotide sequences that encode them are provided herein as SEQ ID NOS: 61, 63, 65, 67, 121, 123, and 125, respectively. Additional non-naturally occurring glucose dehydrogenases that are suitable for use in the practice of the present invention are provided in the patent application entitled, "Improved Glucose Dehydrogenase Polypeptides and Related Polynucleotides," corresponding to Attorney Docket No. 0352.110US/15076US01, filed on August 11, 2003, and assigned U.S. application serial number 60/494,300, and in the patent application entitled, "Improved Glucose Dehydrogenase Polypeptides and Related Polynucleotides," corresponding to Attorney Docket No. 0352.210US/15076US02, filed on February 18, 2004, and assigned U.S. application serial number ______________________________, both of which are incorporated herein by reference in their entireties.

Glucose dehydrogenases employed in the practice of the present invention may exhibit an activity of at least about 10 μmol/min/mg and sometimes at least about 10^2 μmol/min/mg or about 10^3 μmol/min/mg, up to about 10^4 μmol/min/mg or higher in the assay described in Example 4.

When glucose and glucose dehydrogenase are employed as the cofactor regeneration system, as the 4-halo-3-ketobutyric acid ester or amide is reduced by the ketoreductase and NADH or NADPH, the resulting NAD or NADP is reduced by the coupled oxidation of

glucose to gluconic acid by the glucose dehydrogenase. The net reaction is described by equation (6), which is the summation of equations (4) and (5):



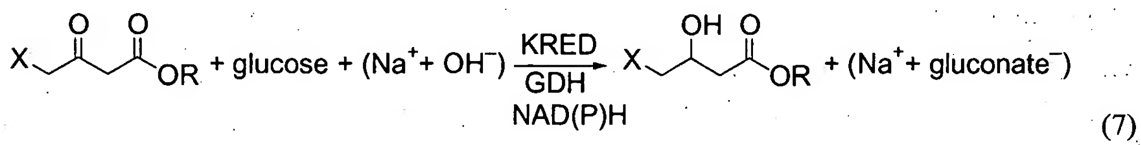
(6)

The ketoreductase-catalyzed reduction of 4-halo-3-ketobutyric acid ester or amide is generally carried out in a solvent. The solvent may be a co-solvent system, such as, for example, an aqueous co-solvent system. Suitable solvents (including co-solvent systems) for carrying out this conversion are the same as those described above for the HHDH-catalyzed conversion of 4-halo-3-hydroxybutyric acid esters and amides to 4-cyano-3-hydroxybutyric acid esters and amides.

The aqueous solvent (water or aqueous co-solvent system) may be pH-buffered or unbuffered. The conversion of the 4-halo-3-ketobutyric acid ester or amide to the 4-halo-3-hydroxybutyric acid ester or amide may be carried out at a pH of about 5 or above. Generally, the conversion is carried out at a pH of about 10 or below, usually in the range of from about 5 to about 10. Typically, the conversion is carried out at a pH of about 9 or below, usually in the range of from about 5 to about 9. Preferably, the conversion is carried out at a pH of about 8 or below, usually in the range of from about 5 to about 8, and more preferably in the range of from about 6 to about 8. Alternatively, the conversion may be carried out at neutral pH, i.e., about 7.

When the glucose/glucose dehydrogenase cofactor regeneration system is employed, the co-production of gluconic acid (pKa = 3.6), as represented in equation (6) causes the pH of the reaction mixture to drop if the resulting aqueous gluconic acid is not otherwise neutralized. The pH of the reaction mixture may be maintained at the desired level by standard buffering techniques, wherein the buffer neutralizes the gluconic acid up to the buffering capacity provided, or by the addition of a base concurrent with the course of the conversion. Suitable buffers and procedures for buffering and suitable bases and procedures for the addition of base during the course of the conversion are the same as those described above for the HHDH-catalyzed conversion of 4-halo-3-hydroxybutyrate esters and amides to 4-cyano-3-hydroxybuyrate esters and amides.

In the ketoreductase-catalyzed reduction of the 4-halo-3-ketobutyric acid ester or amide using glucose/glucose dehydrogenase for cofactor regeneration, when the pH is maintained by buffering or by addition of a base over the course of the conversion, an aqueous gluconate salt rather than aqueous gluconic acid is the product of the overall process. For example, equation (7) represents the overall process when aqueous sodium hydroxide ($Na^+ + OH^-$) is added over the course of the reaction to maintain the pH:



(7)

When base addition is employed to neutralize the gluconic acid released during the ketoreductase-catalyzed reduction of a 4-halo-3-ketobutyric acid ester or amide using the glucose/glucose dehydrogenase cofactor regeneration system, the progress of the conversion may be monitored by the amount of base added to maintain the pH. Typically bases added to unbuffered or partially buffered reaction mixtures over the course of conversion are added in aqueous solutions.

The terms "formate dehydrogenase" and "FDH" are used interchangeably herein to refer to an NAD or NADP-dependent enzyme that catalyzes the conversion of formate and NAD or NADP to carbon dioxide and NADH or NADPH, respectively. Formate dehydrogenases that are suitable for use in the practice of the present invention include both naturally occurring formate dehydrogenases, as well as non-naturally occurring formate dehydrogenases. Formate dehydrogenases include those corresponding to SEQ ID NOS: 70 (*Pseudomonas sp.*) and 72 (*Candida boidinii*), which are encoded by polynucleotide sequences corresponding to SEQ ID NOS: 69 and 71, respectively. Formate dehydrogenases employed in the practice of the present invention, whether naturally occurring or non-naturally occurring, may exhibit an activity of at least about 1 μmol/min/mg, sometimes at least about 10 μmol/min/mg, or at least about 10^2 μmol/min/mg, up to about 10^3 μmol/min/mg or higher, and can be readily screened for activity in the assay described in Example 4.

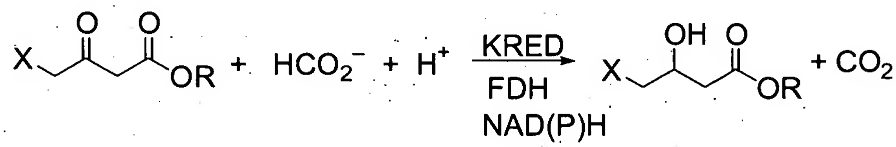
As used herein, the term "formate" refers to formate anion (HCO_2^-), formic acid (HCO_2H), and mixtures thereof. Formate may be provided in the form of a salt, typically an

alkali or ammonium salt (for example, HCO_2Na, $KHCO_2NH_4$, and the like), in the form of formic acid, typically aqueous formic acid, or mixtures thereof. Formic acid is a moderate acid. In aqueous solutions within several pH units of its pKa (pKa = 3.7 in water) formate is present as both HCO_2^- and HCO_2H in equilibrium concentrations. At pH values above about 4, formate is predominantly present as HCO_2^-. When formate is provided as formic acid, the reaction mixture is typically buffered or made less acidic by adding a base to provide the desired pH, typically of about 5 or above. Suitable bases for neutralization of formic acid are as described for neutralization of hydrocyanic acid, above.

For pH values above about 5, at which formate is predominantly present as HCO_2^-, equation (8) describes the formate dehydrogenase-catalyzed reduction of NAD or NADP by formate.

$$HCO_2^- + NAD(P)+ \xrightarrow{FDH} CO_2 + NADP(H) \qquad (8)$$

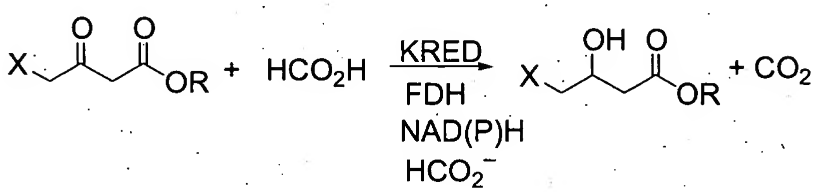
When formate and formate dehydrogenase are employed as the cofactor regeneration system, as the 4-halo-3-ketobutyric acid ester or amide is reduced by the ketoreductase and NADH or NADPH, the resulting NAD or NADP is reduced by the coupled oxidation of formate to carbon dioxide by the formate dehydrogenase. The net reaction is described by equation (9), which is the summation of equations (4) and (8):



(9)

Equation (9) shows that when the formate/formate dehydrogenase cofactor regeneration system is employed for the reduction of the 4-halo-3-ketobutyric acid ester or amide in aqueous solution with pH above about 5, protons in solution are consumed and the reaction causes the pH of the reaction mixture to rise if it is not otherwise buffered or re-acidified. The pH of the reaction mixture may be maintained at the desired level by standard buffering techniques, wherein the buffer releases protons up to the buffering capacity provided, or by the addition of an acid concurrent with the course of the conversion. Suitable acids to add during the course of the reaction to maintain the pH include organic acids, for example carboxylic acids, sulfonic acids, phosphonic acids, and the like, mineral acids, for

example hydrohalic acids (such as hydrochloric acid), sulfuric acid, phosphoric acid, and the like, acidic salts, for example dihydrogenphosphate salts (e.g. KH_2PO_4), bisulfate salts (e.g. $NaHSO_4$) and the like. Particularly preferred is formic acid, whereby both the formate concentration and the pH of the solution are maintained. For example, equation (10) represents the overall process when formic acid (HCO_2H) is added over the course of the reaction to maintain an initial pH above about 5. While the formate is present predominantly as HCO_2^- in the reaction mixture, the HCO_2^- concentration is maintained while the conversion in net consumes the added formic acid.



(10)

When acid addition is employed to maintain the pH during the ketoreductase-catalyzed reduction of a 4-halo-3-ketobutyric acid ester or amide using the formate/formate dehydrogenase cofactor regeneration system, the progress of the conversion may be monitored by the amount of acid added to maintain the pH. Typically, acids added to unbuffered or partially buffered reaction mixtures over the course of conversion are added in aqueous solutions.

In carrying out the methods of the present invention, either the oxidized or reduced form of the cofactor may be provided initially. As described above, the cofactor regeneration system converts oxidized cofactor to its reduced form, which is then utilized in the reduction of the ketoreductase substrate (i.e., 4-halo-3-ketobutyric acid ester or amide) to the corresponding halohydrin.

As with the halohydrin dehalogenases, the ketoreductase and enzymes of the cofactor regeneration system may be provided to the reaction mixture for converting 4-halo-3-ketobutyric acid ester or amide in the form of purified enzyme, cell extract, cell lysate, or whole cells transformed with gene(s) encoding the ketoreductase and enzymes of the cofactor regeneration system. The genes encoding the enzymes can be transformed into host cells either separately, or together into the same host cell. For example, in one embodiment one set of host cells can be transformed with ketoreductase encoding gene(s) and another set can

be transformed with cofactor regeneration system enzyme (e.g., GDH, FDH, and the like) encoding gene(s). Both sets of transformed cells can be utilized together in the reaction mixture in the form of whole cells or cell lysates or cell extract derived therefrom. Alternatively, a host cell can be transformed with genes encoding <u>both</u> ketoreductase and a cofactor regeneration system enzyme, such that each cell expresses both ketoreductase and the cofactor regeneration system enzyme. In a further embodiment, the host cell can be transformed with genes encoding ketoreductase, a cofactor regeneration system enzyme, and a halohydrin dehalogenase. These cells can be utilized in the methods of the present invention to provide the enzymes in the form of whole cells, cell lysate, or cell extract. As described for the reaction mixture of the HHDH-catalyzed method, the solid reactants (i.e., enzymes, salts, cofactor regeneration system, cofactor, and the like) may be provided in a variety of different forms, including powder (e.g., lyophilized, spray dried, and the like), solution, emulsion, suspension, and the like.

The quantities of reactants used in the reduction step will generally vary depending on the quantities of 4-halo-3-hydroxybutyric acid ester or amide desired, and concomitantly the amount of ketoreductase substrate employed. The following guidelines can be used to determine the amounts of ketoreductase, cofactor, and cofactor regeneration system to use. Generally, 4-halo-3-ketobutyric acid esters and amides are employed at a concentration of about 10 to 500 grams/liter using from about 10 mg to about 5 g of ketoreductase and about 25 mg to about 5 g of cofactor. Those having ordinary skill in the art will readily understand how to vary these quantities to tailor them to the desired level of productivity and scale of production. Appropriate quantities of cofactor regeneration system may be readily determined by routine experimentation based on the amount of cofactor and/or ketoreductase utilized. In general, the reductant (e.g. glucose, formate) is utilized at levels above the equimolar level of ketoreductase substrate to achieve essentially complete or near complete conversion of the ketoreductase substrate.

The order of addition of reactants is not critical. The reactants may be added together at the same time to a solvent (e.g., monophasic solvent, biphasic aqueous co-solvent system, and the like), or alternatively, some of the reactants may be added separately, and some together at different time points. For example, the cofactor regeneration system, cofactor, ketoreductase, and ketoreductase substrate may be added first to the solvent

For improved mixing efficiency when an aqueous co-solvent system is used, the cofactor regeneration system, ketoreductase, and cofactor are usually added and mixed into the aqueous phase first. The organic phase may then be added and mixed in, followed by addition of the ketoreductase substrate. Alternatively, the ketoreductase substrate may be premixed in the organic phase, prior to addition to the aqueous phase.

As for the halohydrin dehalogenase-catalyzed conversion of 4-halo-3-hydroxybutyric acid esters and amides, suitable conditions for carrying out the ketoreductase-catalyzed reduction of 4-halo-3-ketobutyric acids esters and amides of the present invention include a wide variety of conditions that can be readily determined by those having ordinary skill in the art. Suitable temperatures for carrying out the ketoreductase-catalyzed reduction step are typically in the range of from about 15°C to about 75°C. Usually, the reactions are carried out at a temperature in the range of from about 20°C to about 55°C, and preferably from about 20°C to about 45°C. The reaction may also be carried out under ambient conditions,

As in the halohydrin dehalogenase-catalyzed reaction, the ketoreductase-catalyzed reaction is allowed to proceed until essentially complete or near complete conversion of substrate is observed using methods that are known in the art. As in the halohydrin dehalogenase-catalyzed reaction, the progression of the ketoreductase-catalyzed reaction may be monitored by monitoring the amount of base or acid added to counter the pH change that may otherwise occur with the particular cofactor regeneration system that is used, as described above.

The ketoreductase-catalyzed reduction of the 4-halo-3-ketobutyric acid ester or amide substrate generates a new stereogenic carbon at the 3-position of the 4-halo-3-hydroxybutyric acid ester or amide product. Typically, the 4-halo-3-hydroxybutyric acid ester or amide is generated with a relatively high stereoselectivity at the 3-position. Thus, the 4-halo-3-hydroxybutyric acid esters and amides generated by the ketoreductase-catalyzed reduction of 4-halo-3-ketobutyric acid esters and amides are typically chiral and non-racemic. The ketoreductase reactions used in the present invention typically generate preferred nonracemic, chiral 4-halo-3-hydroxybutyric acid esters having an e.e. of at least about 90% e.e., usually at least about 95% e.e., and typically at least about 99% e.e. The Examples illustrate embodiments providing ethyl (S)-4-chloro-3-hydroxybutyrate with greater than 99% e.e.

As used herein, the term "enantiomeric excess" or " e.e." refers to the absolute difference between the mole or weight fractions of major ($F_{(+)}$) and minor ($F_{(-)}$) enantiomers

(i.e., $|F_{(+)} - F_{(-)}|$), where $F_{(+)} + F_{(-)} = 1$. Percent e.e. is $100 \times |F_{(+)} - F_{(-)}|$. Enantiomeric composition can be readily characterized by using the gas chromatography method described in Example 6, hereinbelow, and using methods that are known in the art.

As described above, when these nonracemic chiral 4-halo-3-hydroxybutyric acid esters or amides are used as substrates in the halohydrin dehalogenase-catalyzed reactions of the present invention, the resulting 4-substituted-4-hydroxybutyric acid esters or amides are substantially equally nonracemic, with little or no loss in stereopurity. The combination of the high stereoselectivity of the ketoreductase-catalyzed production of the nonracemic 4-halo-3-hydroxybutyric acid esters or amides and the high stereofidelity of the halohydrin dehalogenase-catalyzed conversion of them to the corresponding nonracemic 4-cyano-3-hydroxybutyric acid esters or amides provides a particularly attractive inventive process for the overall production of nonracemic 4-cyano-3-hydroxybutyric acid esters or amides of high e.e. from 4-halo-3-ketobutyric acid esters or amides.

A further significant characteristic of the present invention is that the yield of chiral products generated is very high. Typically, the yields of 4-halo-3-hydroxybutyric acid ester or amide and 4-nucleophile substituted-3-hydroxybutyric acid ester or amide products generated in accordance with the methods of the present invention are at least about 70%, usually at least about 80%, typically at least about 90%, and may be at least about 95%. The computation of product yield is based on initial substrate quantity provided and the amount of product formed in the reaction mixture. Product 4-halo-3-hydroxybutyric acid ester or amide may be optionally purified prior to contacting with the halohydrin dehalogenase. As used herein, the term "purified" refers to a process in which a separation process is applied to a mixture, resulting in an increase in concentration of one component relative to other components in the mixture. Suitable purification processes employed in the practice of the present invention include, for example, filtration, solid or liquid phase extraction, distillation, and the like.

If the 4-halo-3-hydroxybutyric acid ester or amide is purified from the ketoreductase reaction mixture, it is subsequently added to a solvent (e.g., a monophasic solvent, a biphasic aqueous co-solvent system) with the halohydrin dehalogenase and nucleophile.

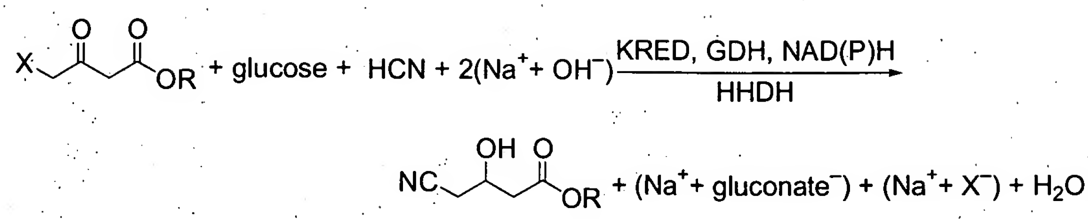
III. ENZYMATIC CONVERSION OF 4-HALO-3-KETOBUTYRIC ACID ESTER/AMIDE TO 4-NUCLEOPHILE SUBSTITUTED-3-HYDROXYBUTYRIC ACID ESTER/AMIDE IN A SINGLE REACTION VESSEL

The present invention provides a method for carrying out the conversion of 4-halo-3-ketobutyric acid esters and amides to the corresponding 4-nucleophile substituted-3-hydroxybutyric acid esters and amides in a single reaction vessel, the method comprising contacting the 4-halo-3-ketobutyric acid ester or amide with a ketoreductase, a cofactor, a cofactor regeneration system, a nucleophile, and a halohydrin dehalogenase to form a reaction mixture for converting the 4-halo-3-ketobutyric acid ester or amide to a 4-nucleophile substituted-3-hydroxybutyric acid ester or amide

Mechanistically, this single-vessel method proceeds via ketoreductase-catalyzed conversion of the 4-halo-3-ketobutyric acid ester or amide to provide the 4-halo-3-hydroxybutyric acid ester or amide *in situ*, and consequent halohydrin dehalogenase-catalyzed conversion of the 4-halo-3-hydroxybutyric acid ester or amide to the corresponding 4-nucleophile substituted-3-hydroxybutyric acid ester or amide. Significantly, the 4-halo-3-hydroxybutyric acid ester or amide produced by the ketoreductase-catalyzed reaction is not separated or recovered prior to its contact with halohydrin dehalogenase and nucleophile (e.g., cyanide and the like) for its conversion to 4-nucleophile substituted-3-hydroxybutyric acid ester or amide.

Suitable reactants (substrates, enzymes, cofactors), solvents, pH, temperature, and other reaction conditions and procedures for the single-vessel conversion of 4-halo-3-ketobutyric acid ester or amide to 4-nucleophile substituted-3-hydroxybutyric acid ester or amide are the same as those described above for the carrying out the halohydrin dehalogenase-catalyzed conversion of 4-halo-3-hydroxybutyric acid esters and amides to the corresponding 4-nucleophile substituted-3-hydroxybutyric acid esters and amides.

When glucose and glucose dehydrogenase are used as the cofactor regeneration system and two equivalents of base are added during the course of the reaction to neutralize both the gluconic acid and hydrohalic acid produced and maintain the initial pH of the reaction mixture (for initial pHs in the range of about 5 to about 9), the overall process in a single-vessel reaction is described by equation (11), which is the summation of equations (2) and (7), wherein aqueous sodium hydroxide is illustrated as the base.



(11)

Other single-vessel overall process equations can result from summing equations describing other options for conducting the halohydrin dehalogenase-catalyzed reaction (e.g.. using a cyanide salt as the base) and/or the ketoreductase reaction (e.g. using formate and formate dehydrogenase as the cofactor regeneration system), as described above for the separately conducted reactions.

It will also be understood that the same single-vessel result may be obtained by first conducting the ketoreductase reaction separately as described above, then subsequently adding halohydrin dehalogenase and cyanide into the ketoreductase reaction mixture and conducting the halohydrin dehalogenase reaction in the presence of the ketoreductase reaction components.

An embodiment of a single-vessel process for converting a 4-halo-3-ketobutyric acid ester to a 4-cyano-3-hydroxybutyric acid ester is illustrated in Example 24.

IV. <u>HALOHYDRIN DEHALOGENASE-CATALYZED CONVERSION OF VICINAL HALO, HYDROXY SUBSTITUTED CARBOXYLIC ACID ESTERS TO VICINAL CYANO, HYDROXY SUBSTITUTED CARBOXYLIC ACID ESTERS</u>

In addition to 4-halo-3-hydroxybutyric acid ester substrates, it has been discovered that halohydrin dehalogenases can be used to catalyze the conversion of other vicinal halo, hydroxy substituted carboxylic acid ester substrates to their corresponding vicinal cyano, hydroxy substituted carboxylic acid esters, using the same conditions as described in part I. Thus, the present invention also provides a method for producing a vicinal cyano, hydroxy substituted carboxylic acid ester from a vicinal halo, hydroxy substituted carboxylic acid ester, the method comprising:

(a) providing a vicinal halo, hydroxy substituted carboxylic acid ester,

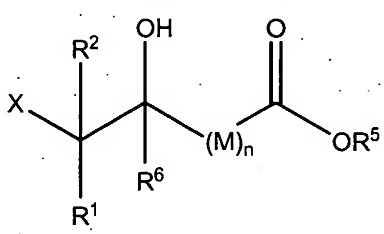
wherein the halo substituent is selected from the group consisting of chlorine, bromine, and iodine; and

(b) contacting the vicinal halo, hydroxy substituted carboxylic acid ester with a halohydrin dehalogenase and cyanide under conditions suitable to form a reaction mixture for converting the vicinal halo, hydroxy substituted carboxylic acid ester to a vicinal cyano, hydroxy substituted carboxylic acid ester.

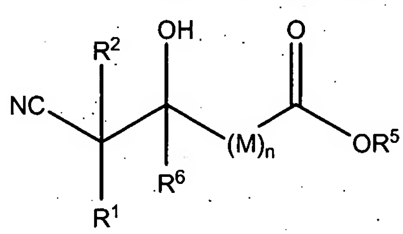
As used herein, the term "vicinal halo, hydroxy substituted" refers to halogen and hydroxyl group substitutions on adjacent carbons. The term "vicinal cyano, hydroxy substituted" is used herein to refer to cyano (C≡N) and hydroxyl groups substitutions on adjacent carbons. Vicinal halo, hydroxyl substituted carboxylic acid esters that are suitable for use in the practice of the present invention include those that are not 4-halo-3-hydroxybutyric acid ester. Concomitantly, vicinal cyano, hydroxy substituted carboxylic acid ester product generated by these methods include those that are not 4-cyano-3-hydroxybutyric acid ester.

Preferred vicinal halo, hydroxy substituted carboxylic acid ester substrates of the present invention are chiral, being stereogenic at the hydroxy-substituted carbon, and may be racemic or non-racemic. In these embodiments, certain halohydrin dehalogenase enzymes used in the process of the present invention convert the chiral vicinal halo, hydroxyl substituted carboxylic acid ester substrate to the corresponding vicinal cyano, hydroxy substituted carboxylic acid ester with retention of the absolute stereochemistry at the stereogenic hydroxy-substituted carbon. Non-racemic chiral vicinal halo, hydroxy substituted carboxylic acid ester substrates may be converted to substantially equally non-racemic vicinal cyano, hydroxy substituted carboxylic acid ester products with little or no loss in stereopurity.

Suitable vicinal halo, hydroxy substituted carboxylic acid esters employed in the practice of the present invention include those having the structure VII, which provide vicinal cyano, hydroxy substituted carboxylic acid ester having the corresponding structure VIII.



VII



VIII

where:

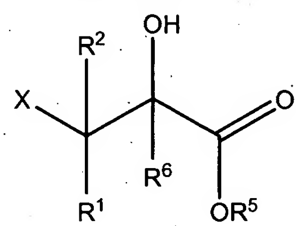
X, R^1, R^2, R^5, and R^6 are as defined for structure IA, and

n is zero or from 1 to 9, inclusive, and

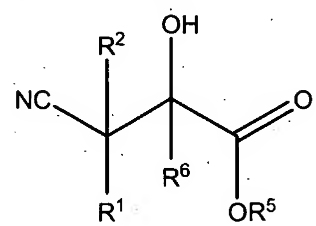
each M_n is independently selected from -C(=O)- (i.e., carbonyl) or $-CR^{n'}R^{n''}-$, wherein $R^{n'}$ and $R^{n''}$ are each independently selected from the group consisting of hydrogen, fluorine, an optionally substituted lower alkyl, an optionally substituted cycloalkyl, an optionally substituted lower alkenyl, an optionally substituted aryl, an optionally substituted arylalkyl, amino, an optionally substituted lower alkylamino, an optionally substituted cycloalkylamino, an optionally substituted lower alkoxy, an optionally substituted cycloalkoxy, an optionally substituted aryloxy, an optionally substituted aryl, hydroxyl, nitro, amino, cyano, carboxy (i.e. a carboxylate or carboxylic acid group), carboalkoxy (i.e. an ester group), carbamide (i.e.an amide group), and acyl (i.e. forming a ketone).

Suitable vicinal halo, hydroxy substituted carboxylic acid esters have the structure VII with n=1, and include the 4-halo-3-hydroxybutyric acid esters previously described.

Other suitable vicinal halo, hydroxy substituted carboxylic acid esters have the structure VII with n=0, thereby having structure IX, which provide vicinal cyano, hydroxy substituted carboxylic acid ester having the corresponding structure X.



IX



X

where:

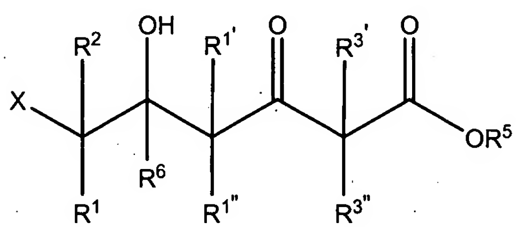
X, R^1, R^2, R^5, and R^6 are as defined for structure VII.

Suitable vicinal halo, hydroxy substituted carboxylic acid esters having the structure IX include 4-halo-2-hydroxypropionic acid esters. With respect to structures VII and IX, X is typically chlorine or bromine, and preferably chlorine. With respect to structures VII through X, R^1, R^2, R^5, and R^6 are preferably each independently either hydrogen or a lower alkyl.

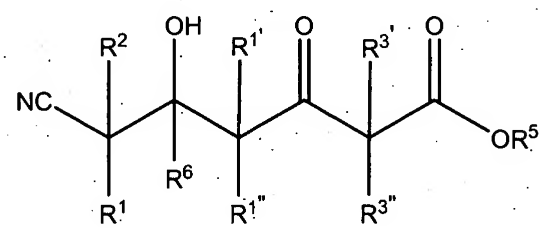
Other suitable vicinal halo, hydroxy substituted carboxylic acid esters have the structure VII with n from 2 to 9, inclusive. Typically, n is from 2 to 8, inclusive, and more typically, from 2 to 7, inclusive, and usually, from 2 to 6, inclusive. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=2 include 5-halo-4-hydroxypentanoic

acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=3 include 6-halo-5-hydroxyhexanioc acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=4 include 7-halo-6-hydroxyheptanoic acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=5 include 8-halo-7-hydroxyoctanoic acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=6 include 9-halo-8-hydroxynonanoic acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=7 include 10-halo-9-hydroxydecanoic acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=8 include 11-halo-10-hydroxyundecanoic acid esters. Suitable vicinal halo, hydroxy substituted carboxylic acid esters having n=9 include 12-halo-11-hydroxydodectanoic acid esters.

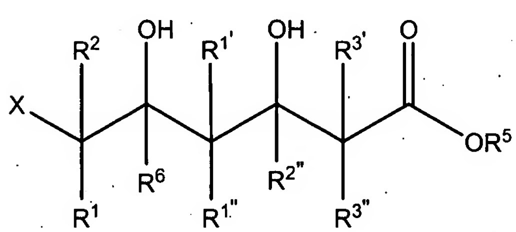
Preferred vicinal halo, hydroxy substituted carboxylic acid esters are 6-halo-5-hydroxy-3-oxohexanoic acid esters having the structure XI and 6-halo-3,5-dihydroxy-hexanoic acid esters having the structure XIII, which provide vicinal cyano, hydroxy substituted carboxylic acid ester having the structures XII and XIV, respectively.



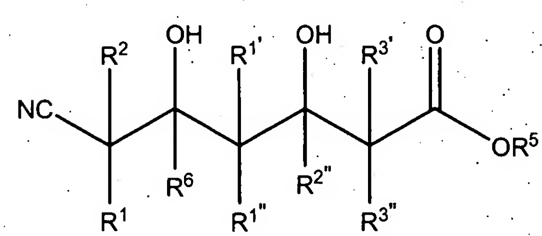
XI



XII



XIII



XIV

where for XI, XII, XIII, and XIV:

X, R^1, R^2, R^5, and R^6 are as defined for structure VII. $R^{1'}$, $R^{1''}$, $R^{2'}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are as defined for $R^{n'}$ and $R^{n''}$ in structure VII. Preferably, R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are each independently either hydrogen or a lower alkyl. X is typically chlorine or bromine.

Preferred 6-halo-5-hydroxy-hexanoic acid esters of structure XI or XIII have X=Cl.

Specific 6-halo-5-hydroxy-3-oxohexanoic acid esters of structure XI that may be employed in the practice of the present invention include t-butyl 6-chloro-5-hydroxy-3-oxohexanoic acid ester (i.e., where X is chlorine, R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are hydrogen and R^5 is t-butyl) and t-butyl 6-bromo-5-hydroxy-3-oxohexanoic acid ester (i.e., where X is bromine, R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are hydrogen, and R^5 is t-butyl). When employed in the method of the present invention, these substrates yield the corresponding vicinal cyano, hydroxyl substituted product having structure XII, where R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are hydrogen and R^5 is t-butyl.

Specific 6-halo-3,5-dihydroxyhexanoic acid esters having the structure XIII that may be employed in the practice of the present invention include t-butyl 6-chloro-3,5-dihydroxy-hexanoic acid ester (i.e., where X is chlorine, R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are hydrogen, and R^5 is t-butyl) and t-butyl 6-bromo-3,5-dihydroxyhexanoic acid ester (i.e., where X is bromine, R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are hydrogen, and R^5 is t-butyl). When employed in the method of the present invention, these 6-halo-3,5-dihydroxyhexanoic acid ester substrates yield vicinal cyano, hydroxyl substituted product having structure XIV, where R^1, R^2, R^6, $R^{1'}$, $R^{1''}$, $R^{2''}$, $R^{3'}$, and $R^{3''}$ are hydrogen, and R^5 is t-butyl

Suitable halohydrin dehalogenases for catalyzing the conversion of vicinal halo, hydroxy substituted carboxlic acid ester substrate to vicinal cyano hydroxyl substituted carboxylic acid product include both naturally occurring and non-naturally occurring halohydrin dehalogenases, as previously described in part I *supra*. Halohydrin dehalogenases may be readily identified using the assay described in Example 4 (part (3)) and substituting for ethyl (S)-4-chloro-3-hydroxybutyrate the vicinal halo, hydroxyl substituted carboxylic acid substrate of interest. Conditions that are suitable to form a reaction mixture for converting the vicinal halo, hydroxy substituted carboxylic acid ester to a vicinal cyano,, hydroxy substituted carboxylic acid ester are the same as described for converting 4-halo-3-hydroxybutyric acid esters to their corresponding 4-cyano-3-hydroxybutyric acid esters (see part I *supra*), e.g., cyanide sources, methods for controlling pH, temperatures, solvents and co-solvent systems, and the like. Exemplary methods for converting 6-halo-5-hydroxy-3-oxohexanoic acid esters (structure XI) and 6-halo-3,5-dihydroxyhexanoic acid esters (structure XIII) to their corresponding vicinal cyano, hydroxy substituted carboxylic acid esters (i.e., structures XII and XIV, respectively) are provided in Examples 32-41 hereinbelow.

V. COMPOSITIONS

The present invention further provides compositions that are useful for the enzymatic conversion of 4-halo-3-hydroxybutyric acid ester or amide to 4-nucleophile substituted-3-hydroxybutyric acid ester or amide. These compositions comprise a halohydrin dehalogenase, a 4-halo-3-hydroxybutyric acid ester or amide, and a nucleophile. In a preferred composition, the nucleophile is cyanide.

In a further embodiment, the present invention provides compositions useful for preparing 4-nucleophile substituted-3-hydroxybutyric acid esters and amides that have a ketoreductase, a cofactor regeneration system, a cofactor, and a halohydrin dehalogenase. These compositions may further include a 4-halo-3-ketobutyric acid ester or amide.

Any of the previously described ketoreductases, components of a cofactor regeneration system, cofactors, halohydrin dehalogenases, 4-halo-3-ketobutyric acid esters or amides, 4-halo-3-hydroxybutyric acid esters or amides, and nucleophiles may be employed in these compositions.

The present invention also provides compositions that are useful for carrying out the conversion of a vicinal halo, hydroxy substituted carboxylic acid ester to a vicinal cyano, hydroxy substituted carboxylic acid ester or amide. These compositions comprise a halohydrin dehalogenase, a cyanide, and a vicinal halo, hydroxyl substituted carboxylic acid ester.

Compositions of the present invention may be in solid (e.g., a powder) or liquid (e.g., solution, emulsion, suspension, and the like) form. For example, the composition may be in the form of a lyophilized or spray dried powder. Alternatively, the composition may further comprise a solvent.

The compositions may further include components for pH control or processability, including, for example, a salt, an acid, a base, a buffer, a solubilizing agent, etc.

VI. HALOHYDRIN DEHALOGENASES, KETOREDUCTASES, AND COFACTOR REGENERATION SYSTEM ENZYMES AND CORRESPONDING POLYNUCLEOTIDES

In addition to the specific enzymes and polynucleotides described herein, those having ordinary skill in the art will recognize that known techniques can be readily applied in

the discovery of both naturally occurring and non-naturally occurring polynucleotides encoding enzymes suitable for use in the practice of the present invention. See, e.g., Ling, et al., "Approaches to DNA mutagenesis: an overview," Anal. Biochem., 254(2):157-78 (1997); Dale, et al., "Oligonucleotide-directed random mutagenesis using the phosphorothioate method," Methods Mol. Biol., 57:369-74 (1996); Smith, "In vitro mutagenesis," Ann. Rev. Genet., 19:423-462 (1985); Botstein, et al., "Strategies and applications of in vitro mutagenesis," Science, 229:1193-1201 (1985); Carter, "Site-directed mutagenesis," Biochem. J., 237:1-7 (1986); Kramer, et al., "Point Mismatch Repair," Cell, 38:879-887 (1984); Wells, et al., "Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites," Gene, 34:315-323 (1985); Minshull, et al., "Protein evolution by molecular breeding," Current Opinion in Chemical Biology, 3:284-290 (1999); Christians, et al., "Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling," Nature Biotechnology, 17:259-264 (1999); Crameri, et al., "DNA shuffling of a family of genes from diverse species accelerates directed evolution," Nature, 391:288-291; Crameri, et al., "Molecular evolution of an arsenate detoxification pathway by DNA shuffling," Nature Biotechnology, 15:436-438 (1997); Zhang, et al., "Directed evolution of an effective fucosidase from a galactosidase by DNA shuffling and screening," Proceedings of the National Academy of Sciences, U.S.A., 94:45-4-4509; Crameri, et al., "Improved green fluorescent protein by molecular evolution using DNA shuffling," Nature Biotechnology, 14:315-319 (1996); Stemmer, "Rapid evolution of a protein in vitro by DNA shuffling," Nature, 370:389-391 (1994); Stemmer, "DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution," Proceedings of the National Academy of Sciences, U.S.A., 91:10747-10751 (1994); WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; and WO 01/75767 . These and other known methods can be readily applied, for example, together with the assays described herein, to identify other ketoreductases, halohydrin dehalogenases, and cofactor regeneration system enzymes having the activities described herein, as well as other desirable properties, e.g., altered temperature and/or pH optimums, solvent resistance, and the like. For example, a ketoreductase may be mutated or evolved to generate libraries that can be screened to identify a ketoreductase having a preference for one cofactor type over another, for example, NAD versus NADP, or vice-versa.

Polynucleic acid sequences encoding the enzymes employed in the present invention may be codon optimized for optimal production from the host organism selected for expression. Those having ordinary skill in the art will recognize that tables and other references providing codon preference information for a wide range of organisms are readily available. See e.g., Henaut and Danchin, "*Escherichia coli* and *Salmonella*," Neidhardt, et al. eds., ASM Press, Washington, D.C. (1996) pp. 2047-2066.

Enzymes employed in the practice of the present invention may be produced by transforming a vector containing a polynucleotide encoding halohydrin dehalogenase, ketoreductase, or a cofactor regeneration system enzyme into a host cell using well known molecular biology techniques. See, e.g., Berger and Kimmel, "Guide to Molecular Cloning Techniques", <u>Methods in Enzymology</u>, Volume 152, Academic Press, Inc., San Diego, CA; Sambrook, et al., "Molecular Cloning—A Laboratory Manual," 2nd Ed., Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; and "Current Protocols in Molecular Biology," F.M. Ausubel, et al., eds., <u>Current Protocols</u>, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (supplemented through 1999). Methods for making the enzymes are illustrated in Examples 1 and 2.

The foregoing and other aspects of the invention may be better understood in connection with the following non-limiting examples.

<u>EXAMPLES</u>

<u>Example 1: Construction of Expression Constructs for Expression of Halohydrin Dehalogenase, Ketoreductase, and Glucose Dehydrogenase,</u>

(1) <u>Halohydrin Dehalogenase (HHDH)</u>

The gene for the halohydrin dehalogenase was codon optimized for expression in *E. coli* based on the amino acid sequence of the halohydrin dehalogenase from *Agrobacterium sp*. The gene was synthesized using 60-mer oligomers, and cloned into expression vector pCK110700 (depicted in Figure 2) under the control of a T5 promoter. The vectors were transformed into *E. coli* TOP10 (Invitrogene, Carlsbad, CA) from which plasmid DNA was prepared using standard methods. The plasmid DNA was then transformed into *E. coli* BL21 (Stratagene, La Jolla, CA), the expression host, using standard methods. Several clones were

found in the expression library that expressed active HHDH. The genes from these clones were sequenced (see SEQ ID Nos: 13 (HHDH.1), 15 (HHDH.2), and 17 (HHDH.16) which encode polypeptide sequences SEQ ID Nos. 14, 16, and 18, respectively).

(2) Ketoreductase (KRED)

The gene for the ketoreductase was codon optimized for expression in *E. coli* based on the amino acid sequence of the ketoreductase from *Candida magnoliae*. The gene was synthesized using 60-mer oligomers, and cloned into the S*fi*I cloning sites of expression vector, pCK110900 (depicted in Figure 3), under the control of a lac promoter and lacI repressor gene. The expression vector contains the p15A origin of replication and the chloroamphenicol resistance gene. The plasmids were transformed into an *E. coli* expression host using standard methods. Several clones were found that expressed active ketoreductase and their genes were sequenced to confirm the DNA sequences (see SEQ ID Nos: 1 (Ketoreductase 1), 3 (Ketoreductase 2), 5 (Ketoreductase 3), and 7 (Ketoreductase 4), which encode for polypeptide sequences SEQ ID Nos. 2, 4, 6, and 8, respectively).

(3) Glucose Dehydrogenase (GDH)

The genes for the glucose dehydrogenase were amplified using the polymerase chain reaction (PCR) from genomic DNA preparations of *Bacillus subtilis* and *Bacillus megaterium*. The primers for the amplification reactions were designed using the published *B. subtilis* and *B. megaterium* glucose dehydrogenase gene sequences, and were as follows:

B. subtilis forward primer (SEQ ID NO: 19) :

5'-GAATTCGCCCATATGTATCCGGATTTAAAAGG-3'

B. subtilis reverse primer (SEQ ID NO: 20):

5'-TGGCCGGATCCTCATTAACCGCGGCCTGCCTGGA-3'

B. megaterium forward primer (SEQ ID NO: 21):

5'-GAATTCGCCCATATGTATAAAGATTTAGAAGG-3'

B. megaterium reverse primer (SEQ ID NO 22):

5'-GGCCGGATCCTCATTATCCGCGTCCTGCTTGGA-3'

The PCR products were cloned into the S*fi*I cloning sites of expression vector, pCK110900 (depicted in Figure 3), under the control of a lac promoter and lacI repressor gene. The expression vector contains the p15A origin of replication and the chloroamphenicol resistance gene. The plasmids were transformed into an *E.coli* expression

host using standard methods. Several clones were found to express active GDH and the genes were sequenced to confirm the sequences (see SEQ ID Nos: 9 (Glucose dehydrogenase S06-3) and 11 (Glucose dehydrogenase M02-6), which encode for polypeptide sequences SEQ ID Nos. 10 and 12, respectively).

(4) Formate Dehydrogenase (FDH)

The genes for the formate dehydrogenase were codon optimized for expression in *E. coli* based on the amino acid sequences of the formate dehydrogenase from *Pseudomonas* species strain 101 (Protein Database Accession ID 2NAD_A) and *Candida boidinii* (Genbank Accession No. CAA09466). The genes were synthesized using 60-mer oligomers, and cloned into the S*fi*I cloning sites of expression vector, pCK110900 (depicted in Figure 3), under the control of a lac promoter and lacI repressor gene. The expression vector contains the p15Aorigin of replication and the chloroamphenicol resistance gene. The plasmids were transformed into an *E. coli* expression host using standard methods. Clones were found that expressed active formate dehydrogenase and the genes were sequenced to confirm the DNA sequences (see SEQ ID NOS: 69 and 71, which encode for polypeptide sequences SEQ ID Nos. 70 and 72, respectively.)

Example 2: Production of Enzyme

(1) HHDH enzyme:

In an aerated agitated fermentor, 10.0L of growth medium containing 0.528g/L ammonium sulphate; 7.5g/L of di-potassium hydrogen phosphate trihydrate; 3.7g/L of potassium dihydrogen phosphate; 2g/L of Tastone-154 yeast extract; 0.05g/L ferrous sulphate; and 3ml/L of a trace element solution containing 2g/L of calcium chloride dihydrate, 2.2g/L of zinc sulfate septahydrate, 0.5g/L manganese sulfate monohydrate, 1g/L cuprous sulfate heptahydrate: 0.1g/l sodium borate decahydrate and 0.5g/L EDTA, was brought to a temperature of 30 °C. The fermentor was inoculated with a late exponential culture of *Escherchia coli* BL21 (Stratagene, La Jolla, CA) equipped with plasmid containing HHDH polynucleotides as described in Example 1, then grown in a shake flask containing LB, 1% glucose (Sigma Chemical Co., St. Louis, MO), and 30μg/ml chloroamphenicol (Sigma Chemical Co., St. Louis, MO) to a starting optical density at 600 nm (OD_{600}) of 0.5 to 2.0. The fermenter was agitated at 500-1500 rpm and air was supplied to the fermentation

vessel at 1.0-15.0 L/min to maintain a dissolved oxygen level of 30% saturation or greater. The pH of the culture was controlled at 7.0 by addition of 20% v/v ammonium hydroxide. After the culture reached an OD_{600} of 40, the temperature was reduced to 25°C and the expression of halohydrin dehalogenase was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (Sigma Chemical Corp., St. Louis, MO) to a final concentration of 1mM. The culture was grown for another 15 hours. After the induction, the cells were harvested by centrifugation and washed with 10 mM potassium phosphate buffer, pH 7.0. The cell paste was used directly in the downstream recovery process or was stored at –80°C until use.

(2) <u>Ketoreductase enzyme</u>:

In an aerated agitated fermentor, 10.0L of growth medium containing 0.528g/L ammonium sulphate, 7.5g/L of di-potassium hydrogen phosphate trihydrate, 3.7g/L of potassium dihydrogen phosphate, 2g/L of Tastone-154 yeast extract, 0.05g/L ferrous sulphate, and 3ml/L of a trace element solution containing 2g/L of calcium chloride dihydrate, 2.2g/L of zinc sulfate septahydrate, 0.5g/L manganese sulfate monohydrate, 1g/L cuprous sulfate heptahydrate, 0.1g/L sodium borate decahydrate and 0.5g/L EDTA, was brought to a temperature of 30 °C.

The fermentor was inoculated with a late exponential culture of *Escherichia coli* W3110 (pCR2-5) grown in a shake flask containing LB, 1% glucose (Sigma Chemical Co., St. Louis, MO), and 30 μg/ml chloroamphenicol (Sigma Chemical Co., St. Louis, MO) to a starting optical density at 600 nm (OD_{600}) of 0.5 to 2.0. The fermentor was agitated at 500-1500rpm and air was supplied to the fermentation vessel at 1.0-15.0 L/min, and the pH of the culture was controlled at 7.0 by addition of 20% v/v ammonium hydroxide. After the culture reached an OD_{600} of 40, the temperature was reduced to 25 °C and the expression of glucose dehydrogenase was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (Sigma Chemical Corp., St. Louis, MO) to a final concentration of 1 mM. The culture was grown for another 15 hours. After the induction, the cells were harvested by centrifugation and washed with 10 mM potassium phosphate buffer, pH 7.0. The cell paste was used directly in the downstream recovery process or was stored at –80 °C until use.

(3) Glucose dehydrogenase enzyme:

In an aerated agitated fermentor, 10.0L of growth medium containing 0.528g/L ammonium sulphate; 7.5g/L of di-potassium hydrogen phosphate trihydrate; 3.7g/L of potassium dihydrogen phosphate; 2g/L of Tastone-154 yeast extract; 0.05g/L ferrous sulphate; and 3ml/L of a trace element solution containing 2g/L of calcium chloride dihydrate, 2.2g/L of zinc sulfate septahydrate, 0.5g/L manganese sulfate monohydrate, 1g/L cuprous sulfate heptahydrate; 0.1g/l sodium borate decahydrate and 0.5g/L EDTA, was brought to a temperature of 30 °C.

The fermentor was inoculated with a late exponential culture of (pGDHS06 or pGDHM02) grown in a shake flask containing LB, 1% glucose (Sigma Chemical Co., St. Louis, MO), and 30 μg/ml chloroamphenicol (Sigma Chemical Co., St. Louis, MO) to a starting optical density at 600 nm (OD_{600}) of 0.5 to 2.0. The fermenter was agitated at 500-1500rpm and air was supplied to the fermentation vessel at 1.0-15.0L/min, and the pH of the culture was controlled at 7.0 by addition of 20% v/v ammonium hydroxide. After the culture reached an OD_{600} of 40, the temperature was reduced to 25 °C and the expression of glucose dehydrogenase was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (Sigma Chemical Corp., St. Louis, MO) to a final concentration of 1mM. The culture was grown for another 15 hours. After the induction, the cells were harvested by centrifugation and washed with 10 mM potassium phosphate buffer, pH 7.0. The cell paste was used directly in the downstream recovery process or was stored at –80 °C until use.

(4) Formate Dehydrogenase

In an aerated agitated fermenter, 10.0L of autoclaved minimal medium containing 3.5g/L of $NaNH_4HPO_4 \cdot 4H_2O$, 7.5g/L of $K_2HPO_4 \cdot 3\ H_2O$, and 3.7g/L of KH_2PO_4 (see Lageveen, et al., 1988, Appl. Environ. Microbiol. 54:2924. (1988)), 2g/L NH_4Cl, 0.528g/L $(NH_4)2SO_4$, pH 7.0, 5 ml/L of R2 trace elements (see Reisenberg, et al. Appl. Microbiol. Biotechnol 1990 34:77), 20ml/L of 10% yeast extract solution in water, 5 ml/L 1 M $MgSO_4$, 40 ml/L of 50% glucose solution in water were added. The temperature of the medium was brought to 30 °C.

Chloroamphenicol was added from a concentrated stock solution, to a final concentration of 30 μg/ml. The fermenter was inoculated with an overnight culture of

Escherichia coli W3110 (pFDHPs3 or PFDHCb13) grown in a shake flask containing the above minimal medium with R2 trace element solution, pH 7.0, 0.2% yeast extract, 1% glucose, and 30 μg/ml chloroamphenicol to a starting optical density at 600 nm (OD_{600}) of 0.04 – 0.1. The air was supplied to the fermentation vessel at 5.0 L/min. the pH of the culture was maintained at 7.0 using a concentrated solution of potassium hydroxide in water. The culture was grown to an OD_{600} of 12-15, at which time a feed solution of 50% glucose, 6% ammonium chloride and 0.5% magnesium sulfate was initiated at a rate that resulted in a final dissolved oxygen concentration of 30-40% of air saturation. The feed pump rate was controlled such that the dissolved oxygen in the fermenter was maintained around 30% at airflow rate of 10 L/min and agitation rate of 600 rpm. After the culture reached an OD_{600} of 15 and had been exposed to the feeding regimen for a few hours, the expression of the formate dehydrogenase was induced by the addition of 1mM of IPTG. The culture was grown for another 8-18 hours before it was harvested by centrifugation.

Example 3: Enzyme Preparation

(1) Ketoreductase

The cell paste was washed by suspending 1 volume wet weight of cell paste in 3 volumes of 100mM Tris/sulfate (pH 7.2) follwed by centrifugation at 5000g for 40 minutes in a Sorval 12BP. The washed cell paste was suspended in 2 volumes of 100mM Tris/sulfate (pH 7.2). The intracellular KRED was released from the cells by passing the suspension through a homogenizer in two passes using a pressure of 14,000 psig for the first pass and 8,000 psig for the second pass. The lysate was warmed to room temperature, then a 10% w/v solution of polyethyleneimine (PEI), pH 7.2, was added to the lysate to a final PEI concentration of 0.75% w/v and stirred for 30 minutes. The treated homogenate was centrifuged at 10,000 rpm in a Beckman lab centrifuge for 60 minutes. The supernatant was decanted and dispensed in shallow containers, frozen at –20 °C and lyophilized.

(2) Glucose Dehydrogenase

The cell paste was washed by suspending 1 volume wet weight of cell paste in 3 volumes of 100mM Tris/sulfate (pH 7.2) followed by centrifugation at 5000g for 40 minutes in a Sorval 12BP. The washed cell paste was suspended in 2 volumes of 100mM Tris/sulfate (pH 7.2). The intracellular HHDH was released from the cells by passing the suspension

through a homogenizer in two passes using a pressure of 14,000 psig for the first pass and 8,000 psig for the second pass. The homogenate was centrifuged at 10,000 rpm in a Beckman lab centrifuge for 60 minutes. The supernatant was decanted and dispensed in shallow containers, frozen at –20 °C and lyophilized.

(3) Halohydrin Dehalogenase

The cell paste was washed by suspending 1 volume wet weight of cell paste in 3 volumes of 100mM Tris/sulfate (pH 7.2) followed by centrifugation at 5000g for 40 minutes in a Sorval 12BP. The washed cell paste was suspended in 2 volumes of 100mM Tris/sulfate (pH 7.2). The intracellular HHDH was released from the cells by passing the suspension through a homogenizer in two passes using a pressure of 14,000 psig for the first pass and 8,000 psig for the second pass. The cell lysate was allowed to cool to 4 °C between passes through the homogenizer. The homogenate was centrifuged at 10,000 rpm in a Beckman lab centrifuge for 60 minutes. The supernatant was decanted and dispensed in shallow containers, frozen at –20 °C and lyophilized to a powder that was stored at –80 °C.

To assess the quality of the preparation after fermentation, cell lysate containing the expressed halohydrin dehalogenase enzyme was assayed according to the following protocol. Approximately 50 μl of clarified cell lysate in 100mM Tris-SO_4, 100mM NaCN, pH 8.0 was mixed with 10mM ethyl-(S)-4-chloro-3-hydroxybutyrate (Sigma Aldrich, St. Louis, MO or prepared in accordance with the ketoreductase-catalyzed methods described herein). The total reaction volume was 0.2 ml. The reaction was incubated at room temperature for 30 min to 1 hour. The reaction was extracted with 7 volumes of ethyl acetate and the organic layer removed to a 1.8 ml GC vial. The organic layer was analyzed by GC for presence of the ethyl-(R)-4-cyano-3-hydroxybutyrate product. The amount of product produced was determined by comparison to a standard curve prepared and analyzed under the same conditions.

(4) Formate Dehydrogenase

Cell lysate containing expressed formate dehydrogenase was prepared by homogenization of cell paste in 1 volume 100 mM triethanolamine (pH 7.0) at 4°C. The cell lysate was allowed to cool to 4°C between passes through the homogenizer. Cell lysate was

clarified by centrifugation at 4°C. The clarified lysate was assayed as described in Example 4.

Example 4: Characterization of Enzyme Activity

(1) Ketoreductase (KRED)

To a solution of ethyl 4-chloro-3-ketobutyric acid ester (10 mM) in 100 mM potassium phosphate buffer (pH 7.0) was added the ketoreductase enzyme as a predissolved solution in the same buffer. The reaction was initiated by addition of NADPH (1 mM final) and the course of reaction was followed by measurement of the decrease of absorbance at 340 nm. This absorbance corresponds to the NADPH concentration. The results were plotted as Absorbance units (NADPH) vs. time, and the slope of the plot determined (Absorbance units/min). The slope of the Absorbance vs. time plot was converted to concentration units using the extinction coefficient of NADPH, and the activity of the ketoreductase was determined in units of µmol (NADPH consumed)/min/mg (total ketoreductase catalyst). The measurement can also be performed using fluorescent detection utilizing an excitation of 340 nm for NADPH with emission measured at 455 nm. Other substrates of interest may be substituted for ethyl 4-chloro-3-keto-butyric acid ester to evaluate ketoreductase activity with respect to other substrates.

(2) Glucose Dehydrogenase (GDH)

To a solution of 50 mM glucose in 100 mM potassium phosphate buffer (pH 7.0) was added the glucose dehydrogenase enzyme as a predissolved solution in the same buffer. The reaction was initiated by addition of NADP (1 mM final) and the course of reaction was followed by measurement of the increase of absorbance at 340 nm or of the fluorescence (excitation 340 nm, emission 455 nm). The results were plotted as Absorbance units (NADPH) vs. time, and the slope of the plot determined (Absorbance units/min). The slope of the Absorbance vs. time plot was converted to concentration units using the extinction coefficient of NADPH (see (1) above), and the activity of the glucose dehydrogenase was determined in units of µmol (NADPH created)/min/mg (total glucose dehydrogenase catalyst).

(3) Halohydrin dehalogenase (HHDH)

To a solution of ethyl (S)-4-chloro-3-hydroxybutyrate (10 mM) in 300 mM potassium phosphate, 300 mM NaCN buffer (pH 8.0) was added the halohydrin dehalogenase enzyme as a predissolved solution in the same buffer. Over time, aliquots of the mixture were withdrawn and extracted with three volumes of ethyl acetate. The organic layer was then analyzed by gas chromatography (GC), as described hereinbelow in Example 6. Samples were taken at various time points, and the peak area of the product cyanohydrin, ethyl (R)-4-cyano-3-hydroxybutyrate, was plotted as a function of time. The peak areas were converted to concentration units using a standard curve that was prepared for the ethyl (R)-4-cyano-3-hydroxybutyrate. Activity of the halohydrin dehalogenase was determined in units of μmol (cyanohydrin produced)/min/mg (total halohydrin dehalogenase catalyst). Other nucleophiles and/or substrates of interest may be substituted to evaluate halohydrin dehalogenase activity with respect to other nucleophiles and/or substrates.

(4) Formate Dehydrogenase

To a solution of 150 mM formate in 100 mM triethanolamine buffer (pH 7.0) was added the formate dehydrogenase enzyme as a predissolved solution in the same buffer. The reaction was initiated by addition of NAD (2 mM final) and the course of reaction was followed by measurement of the increase of absorbance at 340 nm or of the fluorescence (excitation 340 nm, emission 455 nm). The results were plotted as Absorbance units (NADH) vs. time, and the slope of the plot determined (Absorbance units/min). The slope of the Absorbance vs. time plot was converted to concentration units using the extinction coefficient of NADH, and the activity of the formate dehydrogenase was determined in units of μmol (NADH created)/min/mg (total formate dehydrogenase catalyst).

Example 5: Preparation of ethyl (R)-4-cyano-3-hydroxybutyrate from ethyl 4-chloroacetoacetate (via ethyl (S)-4-chloro-3-hydroxybutyrate).

To a well-stirred solution of 100 mM potassium phosphate buffer, 500 mM NaCl, pH 7 (1 L) at room temperature was added glucose (160g, 830 mmoles, 1.1 equiv). To this was added ketoreductase SEQ ID NO: 2 (0.9g), glucose dehydrogenase S06 SEQ ID NO: 10 (0.5 g) and NADP (0.5g) as lyophilized powders. Once dissolved, butyl acetate (500 mL) was added to form an emulsion. To this emulsion was added a solution of ethyl 4-chloroacetoacetate (100g, 608 mmoles) in butyl acetate (500 mL), dropwise over 3 hours.

The pH was maintained between 6.8 and 7 by an automatic titrater that dispensed Na_2CO_3 (2M in water, about 160 mL total). After 40 hours the automated addition of the base had ceased and there was no residual starting material by gas chromatography. The layers were separated, and the aqueous phase was washed with ethyl acetate (500 mL). The combined organics were dried over anhydrous sodium sulfate, filtered and evaporated on a rotary evaporator, to give essentially pure (~97%) ethyl (S)-4-chloro-3-hydroxybutyrate.

To a well stirred solution of ethyl (S)-4-chloro3-hydroxybutyrate (8.25 g, 50 mmoles) in 300 mM potassium phosphate buffer, 300 mM NaCN pH 8.0 (1L) at 30 °C was added halohydrin dehalogenase SEQ ID NO: 14 (9 g) as a lyophilized powder. After fifty seven hours the mixture was washed with ethyl acetate (2 times 250 mL) and the combined organics dried over anhydrous sodium sulfate. The mixture was filtered and evaporated on a rotary evaporator to give essentially pure ethyl (R)-4-cyano-3-hydroxybutyrate, as determined using the gas chromatography method and elution time data described in Example 6, hereinbelow.

This example shows the process of the invention wherein a 4-cyano-3-hydroxybutyric acid ester (ethyl (R)-4-cyano-3-hydroxybutyrate) is produced by contacting a 4-halo-3-hydroxybutyric acid ester (ethyl (S)-4-chloro-3-hydroxybutyrate) with a halohydrin dehalogenase and cyanide (provided by a cyanide salt, NaCN). It further shows the process of the invention wherein the 4-halo-3-hydroxybutyric acid ester is provided by contacting a 4-halo-3-ketobutyric acid ester (ethyl 4-chloroacetoacetate) with a ketoreductase, a cofactor (NADPH, provided as NADP), and a cofactor regeneration system (glucose and glucose dehydrogenase). It further shows the overall production of nonracemic chiral ethyl (R)-4-cyano-3-hydroxybutyrate from achiral ethyl 4-chloroacetoacetate in high e.e. and in high purity without extensive purification procedures.

Example 6: Characterization of Ethyl (R)-4-cyano-3-hydroxybutyrate

The ethyl 4-cyano-3-(R)-hydroxybutyrate produced in Example 5 was analyzed using gas chromatography with flame ionization (FID) detection using an Agilent HP-5 column, 30 m long, 0.25 μm inner diameter, using the following program: 1 minute at 100°C, 5°C/minute for 10 minutes; 25°C/minute for 2 minutes; then 2 minutes at 200°C. Inlet and outlet temperatures were both 300°C, and the flow rate was 2 ml/minute. Under these

conditions, ethyl (R)-4-cyano-3-hydroxybutyrate elutes at 6.25 minutes, ethyl (S)-4-chloro-3-hydroxybutyrate elutes at 4.5 minutes, and ethyl 4-chloroacetoacetate elutes at 4.1 minutes.

Chemical purity of the species was measured using the integrated peak areas from the gas chromatography results.

Enantioselectivity of the halohydrin dehalogenase (HHDH) with respect to ethyl (R)-4-cyano-3-hydroxybutyrate was measured by gas chromatography and FID detection using a Restek gammaDex SA column (30 m long, 0.32 μm inner diameter) using the following program: 25 minutes at 165°C and flow rate at 2 ml/min. Inlet and outlet temperatures were both at 230°C. Under these conditions ethyl (R)-4-cyano-3-hydroxybutyrate elutes at 19.6 minutes and ethyl (S)-4-cyano-3-hydroxybutyrate elutes at 19.2 minutes.

<u>Example 7: Preparation of Ethyl (S)-4-chloro-3-hydroxybutyrate from Ethyl 4-chloro-acetoacetate.</u>

To a 3-necked jacketed 3L flask equipped with a mechanical stirrer and connected to an automatic titrater by a pH electrode and a feeding tube for addition of base, was charged triethanolamine (6.6 mL) and H_2O (492 mL) to make 100 mM triethanolamine solution. The pH was adjusted to 7 with 37% HCl. Then, D-Glucose (125 g) was added. The water circulating to the flask jacket was set to 30 °C. After 10 minutes, ketoreductase SEQ ID NO: 2 (5.7g) and glucose dehydrogenase S06 SEQ ID NO: 10 (3.1 g) powder were added. After 10 minutes, β-NAD (125 mg) was added and the resulting mixture was allowed to stir for 5 minutes. Then, butyl acetate (250 mL) was charged. Using an addition funnel, 2.4 M ethyl 4-chloroacetoacetate (250 mL, 100 g in 167 mL of butyl acetate) was slowly added over 3 hrs. The pH was maintained at 7 by the automatic titrater by the addition of 2 M Na_2CO_3 (152 mL) over 15 hrs. Subsequently, gas chromatography of a reaction sample showed complete conversion to product. Celite (16 g) was added and the reaction mixture was allowed to stir for 10 minutes. The solution was filtered through a celite pad and the organic layer was separated. The aqueous layer was extracted with butyl acetate (2x 200 mL). The organic layers were combined and the solvent removed under vacuum by rotary evaporation to obtain 87 g of the ethyl (S) 4-chloro-3-hydroxybutyrate. The enantiomeric excess was >99%, as determined after its conversion to ethyl (R)-4-cyano-3-hydroxybutyrate in Example 8.

Example 8: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

To a 3-necked jacketed 3L flask equipped with a mechanical stirrer and connected to an automatic titrater by a pH electrode and a feeding tube for addition of base, was charged H_2O (1200 mL), NaCN (37.25 g) and NaH_2PO_4 (125 g) to bring the solution to pH 7. The water circulator was set to 40 °C. After 10 minutes, halohydrin dehalogenase SEQ ID NO: 32 as cell lysate (250 mL) was added. The reaction mixture was allowed to stir for 5 minutes. Using an addition funnel, ethyl (S)-4-chloro-3-hydroxybutyrate (45 g of the material from Example 7) was slowly added over 1 hour. The pH was maintained at 7 by the automatic titrater by the addition of 10 M NaOH (27 mL) over 17 hrs. Subsequently, gas chromatography of a reaction sample showed complete conversion to product. Celite (16 g) was added to the flask, which was then connected to a diaphragm, whose exhaust is bubbled into 5M NaOH (200 mL), to remove HCN. The mixture was heated to 60 °C under 100mm Hg pressure. After 1 hour a submerged air bubbler was added to the solution to aid the removal of the HCN. After 3 hours, an HCN detector indicated less than 5 ppm HCN in the off-gas. The mixture was allowed to cool to room temperature, then filtered through a celite pad. The filtrate was extracted with butyl acetate (3x 800 mL) and the combined organic layers filtered through a pad of activated charcoal. The solvent was removed under vacuum by rotary evaporation to provide 28.5 g of ethyl (R)-4-cyano-3-hydroxybutyrate. The purity was 98% (w/w) by HPLC and the enantiomeric excess was >99% (by chiral GC, the S enantiomer was undetectable).

Example 9: Preparation of Ethyl (S)-4-chloro-3-hydroxybutyrate from Ethyl 4-chloro-acetoacetate.

To a 100 mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base was charged a solution of glucose (7.5 g) in 100 mM triethanolamine pH 7 buffer (25 mL). To this solution was charged ketoreductase SEQ ID NO: 42 (100 mg); 50 mg GDH SEQ ID NO: 66 and NADP (6.25 mg). Butyl acetate (10 ml) was then charged. Then, ethyl 4-chloroacetoacetate (6 g) in butyl acetate (10 mL) was charged. The pH was maintained at 7 by the automatic titrater by the addition of 4M NaOH (7.5 mL) over 7 hrs. A sample of the reaction mixture was extracted with an equal volume

of butyl acetate and the organic layer was analyzed by GC. The analysis showed 99% conversion of the ethyl 4-chloroacetoacetate to ethyl (S)-4-chloro-3-hydroxybutyrate.

Example 10: Preparation of Ethyl (S)-4-chloro-3-hydroxybutyrate from Ethyl 4-chloroacetoacetate.

The procedure was identical to Example 9 with the exceptions that 400 mg of the ketoreductase SEQ ID NO: 42 was used and NAD+ (12.5 mg) was added in place of the NADP. The addition of the NaOH solution by the automatic titrater was complete in 11 hours and the GC analysis showed 99% conversion of the ethyl 4-chloroacetoacetate to ethyl (S)-4-chloro-3-hydroxybutyrate.

Example 11: Preparation of Ethyl (S)-4-chloro-3-hydroxybutyrate from Ethyl 4-chloroacetoacetate.

To a 100 mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base was charged a solution of glucose (12. g) in water (30 mL). To this solution was charged ketoreductase SEQ ID NO: 42 (100 mg); 50 mg GDH SEQ ID NO: 66 and NADP (6.25 mg). Butyl acetate (10 ml) was then charged. Ethyl 4-chloroacetoacetate (10 g) was then charged via syringe pump as follows: 1 mL was charged rapidly and the remainder was then charged at a rate of 1 mL/hr). The pH was maintained at 7 by the automatic titrater by the addition of 4M NaOH over 18 hours hrs. The stirring was stopped and the phases allowed to separate. The organic layer included some emulsion. The organic layer, including some emulsion, was separated and washed with 10 mL of water. The combined aqueous layers were extracted twice with 20 mL of butyl acetate. The organic extracts were combined and rotary evaporated under vacuum to remove water. Additional butyl acetate was added during the evaporation to help remove the water. When the water was removed the butyl acetate solution was decanted from solids in the flask. Evaporation of the solvent under vacuum then gave 8.85 g of ethyl (S)-4-chloro-3-hydroxybutyrate (87.4% yield) of very good purity.

Example 12: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

To a 170mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base was charged NaCN (1.5 g, 31 mmol) and water (50 mL). The vessel

was sealed and the headspace was deaerated with nitrogen. The pH was adjusted to 7 by the addition of conc. H_2SO_4 (0.9 mL). The reaction mixture was heated to 40 °C and treated with a solution of halohydrin dehalogenase SEQ ID NO: 32 (1.2 g in 10 mL water containing 42 μL of 14M β-mercaptoethanol). Then, ethyl (S)-4-chloro-3-hydroxybutyrate (1.8 g, 10.8 mmol) was added via syringe. The automatic titrater maintained the pH at 7 by the addition of 2M NaOH. After 15 hr the reaction was complete and a total of 4.6 mL 2M NaOH had been added. A sample of the reaction mixture was extracted with an equal volume of butyl acetate. GC analysis of the organic extract showed the conversion of the ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate was >99%.

Example 13: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

The procedure was identical to Example 12 with the exception that 4M NaCN was used as the base instead of the 2M NaOH. After 8 hrs, the reaction was complete and a total of 2.3 mL 4M NaCN had been added. By GC analysis, the conversion of the ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate was >99%.

This example shows the process of the invention using an alkali cyanide as base to maintain both the pH and the cyanide concentration of the reaction mixture constant.

Example 14: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

To a 250 mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base (7.5 M NaOH) was charged water (83.5 mL) and 0.7 g of halohydrin dehalogenase SEQ ID NO: 24. The mixture was stirred for 30 minutes. The titrater was activated and set to maintain pH 7. Then, 25% aqueous HCN (9.26 ml, 8.6 g) was charged over 20 minute to make a 2.3% HCN solution. The mixture was heated at 40 °C for 10 minutes, then ethyl (S)-4-chloro-3-hydroxybutyrate (5 g) was charged over 1 hour. The automatic titrater maintained the pH at 7 by the addition of 2M NaOH. After 20 hrs, GC analysis of a butyl acetate extract of a reaction sample showed the conversion of the ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate was 95%.

This example shows the process of the invention using aqueous hydrocyanic acid as the source of cyanide.

Example 15: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

To a 20 mL screw-cap vial was added NaCN (250 mg) and NaH_2PO_4 (830 mg). Water (10 mL) was added followed by halohydrin dehalogenase SEQ ID NO: 32 as lyophilized powder (200 mg). Then ethyl (S)-4-chloro-3-hydroxybutyrate (300 mg) was added. The vial was capped and heated in an oil bath at 40 °C. After 4 hours, GC analysis of a butyl acetate extract of a reaction sample extract showed of 54% conversion of the ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate. After 72 hrs, the GC analysis showed complete conversion.

Example 16: Preparation of Ethyl (S)-4-cyano-3-hydroxybutyrate from Ethyl (R)-4-chloro-3-hydroxybutyrate

The procedure was identical to that of Example 15 with the exceptions that the (R)-enantiomer of the Ethyl 4-chloro-3-hydroxybutyrate was reacted instead of the (S)-enantiomer and the quantities of all reaction components were halved. After 1 hour reaction time, the GC analysis showed 55% conversion of the ethyl (R)-4-chloro-3-hydroxybutyrate to ethyl (S)-4-cyano-3-hydroxybutyrate.

This example in combination with preceding examples shows that the process of the invention may be used to convert either enantiomer of the 4-halo-3-hydroxybutyric acid ester to the corresponding enantiomer of the 4-cyano-3-hydroxybutyric acid ester.

Example 17: Preparation of Methyl (S)-4-chloro-3-hydroxybutyrate from Methyl 4-chloro-acetoacetate

The procedure was identical to that of Example 9 with the exceptions that an equimolar amount of methyl 4-chloroacetoacetate was reacted instead of the ethyl 4-chloroacetoacetate and the enzymes used were ketoreductase SEQ ID NO: 50 and glucose dehydrogenase SEQ ID NO: 62. The reaction was completed in 11 hrs and the GC analysis showed >99% methyl (S)-4-chloro-3-hydroxybutyrate. The product was isolated by extraction into butyl acetate and solvent evaporation and its identity confirmed by ^1H and ^{13}C NMR.

Example 18: Preparation of Methyl (R)-4-cyano-3-hydroxybutyrate from Methyl (S)-4-chloro-3-hydroxybutyrate

The procedure was identical to that of Example 16 with the exception that an equimolar amount of methyl (S)-4-chloro-3-hydroxybutyrate (prepared by Example 17) was reacted instead of ethyl (R)-4-chloro-3-hydroxybutyrate. After 1 hour reaction time, the GC analysis showed 38% conversion of the methyl (R)-4-chloro-3-hydroxybutyrate to methyl (S)-4-cyano-3-hydroxybutyrate. The product was characterized by ^{1}H and ^{13}C NMR.

Example 19: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-bromo-3-hydroxybutyrate.

The procedure was identical to that of Example 16 with the exception that an equimolar amount of ethyl (S)-4-bromo-3-hydroxybutyrate was reacted instead of ethyl (R)-4-chloro-3-hydroxybutyrate. After 1 hour reaction time, the GC analysis showed 90% conversion of the ethyl (S)-4-bromo-3-hydroxybutyrate to ethyl (S)-4-cyano-3-hydroxybutyrate. The product was characterized by ^{1}H and ^{13}C NMR.

This example shows that the process of the invention wherein the halo substituent of the 4-halo-3-hydroxybutyric acid ester is bromine.

Example 20: Preparation of Ethyl 3-hydroxybutyrate from Ethyl acetoacetate.

The procedure was identical to that of Example 17 with the exceptions that an equimolar amount of ethyl acetoacetate was reacted instead of the methyl 4-chloroacetoacetate and 200 mg of ketoreductase SEQ ID NO: 50 and 100 mg of glucose dehydrogenase SEQ ID NO: 62 were used. The reaction was completed in 6 hrs. The product was isolated by extraction into butyl acetate and solvent evaporation and characterized by ^{1}H and ^{13}C NMR.

In combination with preceding examples, this example demonstrates that ketoreductase enzymes that have activity for the reduction of ethyl acetoacetate to ethyl 3-hydroxybutyrate are useful for the reduction 4-halo-3-ketobutyric acid esters to 4-halo-3-hydroxybutyric acid esters in embodiments of this invention.

Example 21: pH profiles of enzymatic and nonenzymatic test reactions of ethyl 4-chloro-3-hydroxybutyrate with cyanide

Aqueous solutions containing 25 mg/mL sodium cyanide were prepared at pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, and 9.0 by the addition of 85% phosphoric acid while monitoring with pH meter. 5 mL of each solution was charged to a separate 20 mL screw cap vial. Halohydrin dehalogenase SEQ ID NO: 38 (20 mg) was added to each vial, followed by ethyl (S)-4-chloro-3-hydroxybutyrate (50 mg, 0.30 mmoles). For nonenzymatic reactions experiments, the procedure was identical with the exception that the enzyme was omitted. The vials were capped and heated in an oil bath at 55 °C for 3 hrs, then removed and cooled to room temperature. A 0.4 mL sample of each reaction mixture was extracted with 1 mL butyl acetate and the extracts were analyzed by gas chromatography.

The analyzed amounts of substrate and products in each vial are given in Table I, and graphed vs. pH in Figure 1. In both, chlorohydrin means ethyl (S)-4-chloro-3-hydroxybutyrate, cyanohydrin means ethyl (R)-4-cyano-3-hydroxybutyrate, and crotonate means ethyl 4-hydroxycrotonate. In the Table, ND means not detected.

Table I: Millimoles chlorohydrin, cyanohydrin and crotonate by-product analyzed in test reactions with and without halohydrin dehalogenase. See Example 21

	without halohydrin dehalogenase			with halohydrin dehalogenase		
PH	mmol chlorohydrin	mmol cyanohydrin	mmol crotonate	mmol chlorohydrin	mmol cyanohydrin	mmol crotonate
5.0	0.33	ND	ND	0.27	ND	ND
6.0	0.29	ND	ND	0.07	0.20	ND
7.0	0.30	ND	ND	0.01	0.28	ND
7.5	0.31	ND	ND	0.004	0.30	ND
8.0	0.30	0.01	ND	0.002	0.29	ND
8.5	0.21	0.05	0.001	0.001	0.24	ND
9.0	0.11	0.10	0.002	0.001	0.21	ND

The pHs of the final test reaction mixtures were remeasured. For the mixtures including halohydrin dehalogenase with initial pHs of 7 or above (being the mixtures in which near complete conversion of the chlorohydrin to the cyanohydrin occurred, the final mixture pHs were 0.4 to 0.6 pH units below the initial pHs. The other mixtures showed much lesser changes in pH from their initial values.

These data show that under these reaction conditions and time, no measurable nonenzymatic reaction of the ethyl 4-chloro-3-hydroxybutyrate with cyanide occurred at any tested pH less than 8. At pH 8 and above, increasing nonenzymatic reaction with cyanide to form ethyl 4-cyano-3-hydroxybutyrate occurred with increasing pH and was accompanied by increasing formation of ethyl 4-hydroxycrotonate by-product. In contrast, the enzymatic reaction with halohydrin dehalogenase occurred at all the tested pH's greater than 5 and with no detectable formation of ethyl 4-hydroxycrotonate at any tested pH. Additionally, for both enzymatic and nonenzymatic test reactions at pH greater than 8, the mole total of the GC-analyzed products decreased from the initial 0.30 mmoles provided (as ethyl 4-chloro-3-hydroxybutyrate reactant) indicating the increasing formation of non-analyzable by-products with increasing pH greater than 8. It was separately established that the ester group of the reactant and product are increasingly hydrolyzed to carboxylic acid groups at pHs greater than 8 and that the resulting carboxylic acids are not extracted in to the extracts of reaction mixture samples that are analyzed by GC. See Example 22.

Example 22: Nonenzymatic hydrolysis of ethyl 4-cyano-3-hydroxybutyrate.

Aqueous phosphate solutions were prepared at pH 7.0, 7.5, 8.0, 8.5, and 9.0 by dissolving 0.48 g of NaH_2PO_4 in 40 mL water and adjusting the pH by addition of 2M NaOH while monitoring with pH meter. 5 mL of each solution was charged to a separate 20 mL screw cap vial. Then, ethyl (R)-4-cyano-3-hydroxybutyrate (46 mg, 0.29 mmol) was added. The vials were capped and heated in an oil bath at 55 °C for 3 hrs, then cooled to room temperature. A 0.4 mL of each reaction mixture was extracted with 1 mL butyl acetate and the extracts were analyzed by GC. For an external standard a duplicate of the pH 7.0 mixture was freshly prepared and immediately extracted. The analyzed amounts of ethyl 4-cyano-3-hydroxybutyrate in each vial are given in Table II. No product of its hydrolysis was detected in the reaction sample extracts. It was separately established that the carboxylic acid product

of hydrolysis of this ester is not extracted into the extracts of the reaction samples that are analyzed by GC.

Table II: Millimoles chlorohydrin and cyanohydrin analyzed in test hydrolysis reactions. See Example 22

pH	mmol cyanohydrin
7.0	0.29
7.5	0.28
8.0	0.27
8.5	0.26
9.0	0.24

The pHs of the final test mixtures were remeasured. The mixtures with initial pHs of 8.0, 8.5, and 9.0 each had a final pH of 7.4. The mixture with an initial pH of 7.5 had a final pH of 7.3, and the mixture with an initial pH of 7 was unchanged. This evidences the production of carboxylic acid in the higher pH samples causing neutralization of the solutions into the phosphate buffering range.

This example in combination with Example 21 shows that ethyl 4-cyano-3-hydroxy-butyrate is increasingly hydrolyzed with increasing pH at the pHs greater than 8 where it can be produced by nonenzymatic reaction of ethyl 4-chloro-3-hydroxybutyrate with cyanide.

Example 23: Preparation of ethyl (R)-4-cyano-3-hydroxybutyrate from ethyl 4-chloroacetoacetate (via ethyl (S)-4-chloro-3-hydroxybutyrate).

To a 100 mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base (4M NaOH) was charged a solution (25 mL) of glucose (7.5 g) in 100 mM triethanolamine buffer, pH 7. To this solution was charged ketoreductase SEQ ID NO: 50 (50 mg), glucose dehydrogenase SEQ ID NO: 62 (20 mg) and NADP (1.5 mg). Butyl acetate (10 ml) and ethyl 4-chloroacetoacetate (6 g) in additional butyl acetate (10 mL) were then charged. The pH was maintained at 7 by the automatic titrater by the addition of 4M NaOH to the stirring mixture over 13 hrs. The phases were then allowed to separate for 30

minutes and the organic layer (25 mL), containing the ethyl (S)-4-chloro-3-hydroxybutyrate intermediate, was removed.

To a 170 mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base (2M NaOH) was charged sodium cyanide (1.5 g) followed by water (50 mL). The vessel was sealed and the headspace was deaerated with nitrogen. The pH was adjusted to 7 using concentrated sulfuric acid (0.9 mL). The mixture was heated to 40 °C and treated with a solution of halohydrin dehalogenase SEQ ID NO: 32 (1.2 g) in 10 mL water containing 42 uL of 14M β-mercaptoethanol). Then, the organic layer (25 mL) containing ethyl (S)-4-chloro-3-hydroxybutyrate from the first step was added via syringe. The pH was maintained at 7 by the automatic titrater by the addition of 2M NaOH to the stirring mixture. After 15 hr, the conversion of ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate was 33% as indicated by the cumulative addition of 5 mL of the base (15 mL expected for complete conversion).

Example 24: Preparation of ethyl (R)-4-cyano-3-hydroxybutyrate from ethyl 4-chloroacetoacetate (via ethyl (S)-4-chloro-3-hydroxybutyrate).

To a 20 mL screw cap vial was added NaCN (125 mg, 2.55 mmol), NaH_2PO_4 (415 mg, 3.46 mmol) and glucose (750 mg, 3.8 mmol). Water (5 mL) was added followed by NADP (2 mg), ketoreductase SEQ ID NO: 56 (50 mg), glucose dehydrogenase SEQ ID NO: 62 (50 mg), and halohydrin dehalogenase SEQ ID NO: 32 (100 mg). Then ethyl 4-chloro-acetoacete (24 mg, 0.15 mmol) in 0.5 mL butyl acetate was added. The vial was capped and heated in an oil bath at 30 °C. After 1 hr, GC analysis of a butyl acetate extract of a reaction sample showed 100% conversion of the ethyl 4-chloro-acetoacete to ethyl (S)-4-chloro-3-hydroxybutyrate, at 96% selectivity, and ethyl (R)-4-cyano-3-hydroxybutyrate at 4% selectivity. Then, the reaction vial was heated to 40 °C for 15 hrs. GC analysis of a butyl acetate extract then showed 2% of the ethyl (S)-4-chloro-3-hydroxy-butyrate remaining, with overall 98% yield of ethyl (R)-4-cyano-3-hydroxybutyrate based on the starting ethyl 4-chloroacetate.

This example shows the process of the invention wherein a 4-cyano-3-hydroxybutyric acid ester (ethyl 4-cyano-3-(R)-hydroxybutyrate) is produced, via an intermediate 4-halo-3-hydroxybutyric acid ester (ethyl 4-chloro-3-(S)-hydroxybutyrate), by contacting a 4-halo-3-ketobutyric acid ester (ethyl 4-chloroacetoacetate) with a ketoreductase, a cofactor (NADPH,

provided as NADP) a cofactor regeneration system (glucose and glucose dehydrogenase), a halohydrin dehalogenase, and cyanide (provided by an cyanide salt, NaCN) with all the reactants simultaneously present in the reaction mixture.

Examples 25-29: Preparations of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

For each of Examples 25-29, to a 170mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base was charged NaCN (1.5 g, 31 mmol) and water (50 mL). The vessel was sealed and the pH was adjusted to 7 by the addition of conc. H_2SO_4 (0.9 mL). The reaction mixture was heated to 40 °C and treated with a solution of halohydrin dehalogenase (0.4 g in 10 mL water). The halohydrin dehalogenases used for these Examples had the polypeptide sequences given for the following SEQ ID NOs.:

Example 25 SEQ ID No. 32
Example 26 SEQ ID No. 94
Example 27 SEQ ID No. 96
Example 28 SEQ ID No. 98
Example 29 SEQ ID No. 100

Then, ethyl (S)-4-chloro-3-hydroxybutyrate (5.00 g, 30.1 mmol) was added via syringe. The automatic titrater maintained the pH at 7 by the addition of 4M NaCN. The progress of the reactions were monitored by recording the cumulative volume of the NaCN solution added vs. time.

Figure 4 shows the percent conversion of ethyl (S)-4-chloro-3-hydroxybutyrate (calculated from the cumulative equivalents of NaCN added) vs. time for each of these Examples. Example 25 used a halohydrin dehalogenase having the amino acid sequence SEQ ID NO. 32, which it the amino acid sequence of the native halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 (*hheC*), expressed from novel nucleic acid corresponding to SEQ ID NO: 31. Comparison of the percent conversion vs. time for Examples 26 through 29 to that of Example 25 shows that novel halohydrin dehalogenases of the present invention have greater activity than the native halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 (*hheC*).

Example 30: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate

The procedure was identical to that of Examples 25-29 with the exception that halohydrin dehalogenase SEQ ID NO. 104 was used. After 12 hours, the reaction was complete based the addition of the NaCN solution, and GC analysis showed that conversion of the ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate was >99%. The reaction mixture was cooled and combined with five other similarly produced final reaction solutions in a 500 mL three-neck flask. The flask was equipped with a condenser and nitrogen dip tube extending into the liquid, and was connected to a diaphragm pump to apply vacuum (500 mmHg). A caustic trap was used to trap the HCN in the off gas. The reaction mixture was heated to 50 °C and a nitrogen bleed was used to facilitate the removal of HCN. After three hours, the HCN removal was complete. The mixture was cooled and partitioned into centrifuge bottles. 300 mL of butyl acetate was partitioned among the bottles, which were capped and shaken for extraction. The mixtures were centrifuged at 8000 rpm for 30 minutes. The layers were separated and the aqueous phase was likewise extracted two more times with 300 mL of butyl acetate. The combined butyl acetate extracts were evaporated under vacuum to give 19.6 g of ethyl R-4-cyano-3-hydroxybutrate (83% yield) as a pale yellow liquid.

Example 31: Preparation of Ethyl (R)-4-cyano-3-hydroxybutyrate from Ethyl (S)-4-chloro-3-hydroxybutyrate.

To a 170mL vessel connected to an automatic titrater by a pH electrode and a feeding tube for addition of base was charged NaCN (1.5 g, 31 mmol) and water (50 mL). The vessel was sealed and the pH was adjusted to 7 by the addition of conc. H_2SO_4 (0.9 mL). The reaction mixture was heated to 40 °C and treated with a solution of halohydrin dehalogenase SEQ ID NO. 100 (0.4 g in 10 mL water). Then, ethyl (S)-4-chloro-3-hydroxybutyrate (10.0 g, 60.2 mmol) was added via syringe. The automatic titrater maintained the pH at 7 by the addition of 4M NaCN. After 18 hours, the reaction was complete based the addition of the NaCN solution (no more based being added, 14.6 mL cumulatively added) and GC analysis showed that conversion of the ethyl (S)-4-chloro-3-hydroxybutyrate to ethyl (R)-4-cyano-3-hydroxybutyrate was >99%. The reaction mixture was cooled and transferred to a 250 mL three-neck flask. The flask was equipped with a condenser and nitrogen dip tube extending

into the liquid, and was connected to a diaphragm pump to apply vacuum (500 mmHg). A caustic trap was used to trap the HCN in the off gas. The reaction mixture was heated to 50 °C and a nitrogen bleed was used to facilitate the removal of HCN. After three hours, the HCN removal was complete. The mixture was cooled and transferred to a centrifuge bottle. 60 mL of butyl acetate was added to the bottle, which was capped and shaken for extraction. The mixture was centrifuged at 10,000 rpm for 30 minutes. The layers were separated and the aqueous phase was likewise extracted two more times with 60 mL of butyl acetate. The combined butyl acetate extracts were evaporated under vacuum to give 7.7 g of ethyl R-4-cyano-3-hydroxybutrate (81% yield).

Examples 32-36: Preparations of t-Butyl (R)-6-cyano-5-hydroxy-3-oxohexanoate from t-Butyl (R)-6-chloro-5-hydroxy-3-oxohexanoate.

An aqueous solution containing 25 mg/mL sodium cyanide at pH 7.2 was prepared at by dissolving 1.25 g NaCN and 4.2 g NaH_2PO_4 in 50 mL of water. For each of Examples 32-36, 1 mL of the solution was charged to a 5 mL screw cap vial. Halohydrin dehalogenase powder (20 mg) was added to the vial, followed by 10 mg *tert*-butyl-(*S*)-6-chloro-5-hydroxy-3-oxo-hexanoate (Julich Fine Chemicals). The vial was capped and the reaction was stirred at room temperature for 12 hours. The reaction mixture was then extracted with 1 mL MTBE and the organic layer was analyzed by HPLC. In each Example, the *tert*-butyl (R)-6-chloro-5-hydroxy-3-oxohexanoate was completely reacted. The polypeptide sequence of the halohydrin dehalogenase used for each Examples and the analyzed reaction yield of *tert*-butyl (R)-6-cyano-5-hydroxy-3-oxohexanoate were as follows:

Example 32	SEQ ID No.106	25%
Example 33	SEQ ID No. 108	15%
Example 34	SEQ ID No. 32	15%
Example 35	SEQ ID No. 74	10%
Example 36	SEQ ID No. 110	10%

Examples 37-41: Preparations of t-Butyl (3*R*,5*R*)-6-cyano-3,5-dihydroxyhexanoate from t-Butyl (3*R*,5*S*)-6-chloro-3,5-dihydroxy-3-hexanoate.

An aqueous solution containing 28 mg/mL sodium cyanide at pH 6.7 was prepared at by dissolving 1.4 g NaCN and 6 g NaH_2PO_4 in 50 mL of water. For each of Examples 37-41, 1 mL of the solution was charged to a 5 mL screw cap vial. Halohydrin dehalogenase powder (20 mg) was added to the vial, followed by 20 mg *tert*-butyl (3*R*,5*S*)-6-chloro-3,5-dihydroxyhexanoate (Chemistry--A European Journal (2001), 7(21), 4562-4571). The vial was capped and the reaction was stirred at room temperature for 17 hours. The reaction mixture was then extracted with 1 mL ethyl acetate and the organic layer was analyzed by HPLC. In each Example, only t-butyl (3*R*,5*R*)-6-cyano-3,5-dihydroxyhexanoate-and unreacted t-butyl (3*R*,5*S*)-6-chloro-3,5-dihydroxy-3-hexanoate were detected. The polypeptide sequence of the halohydrin dehalogenase used for each Examples and the reaction yield of t-butyl (3*R*,5*R*)-6-cyano-3,5-dihydroxyhexanoate were as follows:

Example 37	SEQ ID No. 100	83%
Example 38	SEQ ID No. 108	65%
Example 39	SEQ ID No. 32	64%
Example 40	SEQ ID No. 74	63%
Example 41	SEQ ID No. 102	81%

In a control reaction, omitting halohydrin dehalogenase, the conversion was 4%.

All publications, patents, patent applications, and other documents cited in this application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

While preferred embodiments of the invention have been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.